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GROWTH AND DEVELOPMENT OF RHESUS MONKEYS EXPOSED TO ELF ELECTRIC AND MAGNETIC FIELDS DURING THE FIRST 54 MONTHS OF LIFE

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May 1986

NAVAL AEROSPACE MEDICAL RESEARCH LABORATORY
PENSACOLA, FLORIDA

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FOREWORD

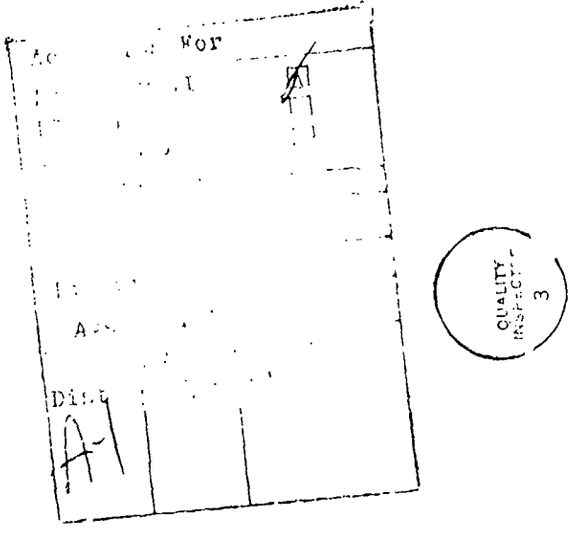
ELF COMMUNICATIONS PROGRAM MANAGER'S PERSPECTIVE

This report on the nearly-continuous exposure of young primates to electromagnetic fields for 4.5 years makes an important contribution to the continuing evaluation of potential biological and health questions related to the U. S. Navy's ELF Communication System.

The accepted scientific and medical practice of using pri[illegible] as human surrogates in laboratory research has been followed by the Navy to obtain data for evaluating the question of human health effects. No biologically -- or medically -- adverse effects have been found from this research.

One of the most important factors in scientific research is the test for the statistical significance of observed results. The results reported herein that could infer important biological effects from continuous exposure to ELF electromagnetic fields in soil, orders of magnitude higher than produced by the ELF Communications System, were not statistically significant. The results are therefore consistent with the current evaluation that the Navy's ELF Communications Systems is unlikely to produce biological or health effects.

NOTE: There are no prohibitions on public access to the ELF Communications System antenna right-of-way or antenna ground terminals in the Chequamegon National Forest in Wisconsin or in the Copper Country and Escanaba State Forests in Michigan. This policy of open access means that people using forest recreational facilities, wildlife, and local biota will occasionally encounter the low-level electromagnetic fields produced by the system. The nominal values of these fields are 0.14 volt/meter and 0.06 gauss in Earth under antennas in Wisconsin, and half those values under the antennas in Michigan. In addition, electric fields are produced in air near the antennas, and have maximum values of 157 volts/meter or less. These fields are comparable in many ways with those produced by commercial power distribution lines serving consumer's residence, the principal difference being the modulated frequency of the ELF system (40-4[illegible] Hz) or (72-80 Hz) versus the unmodulated frequency of power lines (60 Hz).



SUMMARY PAGE

THE PROBLEM

The Department of the Navy is currently completing an Extremely Low Frequency (ELF) submarine communications system in Wisconsin and Michigan. An ecological and health assessment of exposure to ELF electromagnetic fields has been conducted concurrently with the development of this system. An integral part of this assessment was performed at the Naval Aerospace Medical Research Laboratory (NAMRL). In an initial study, rhesus monkeys were exposed at NAMRL for nearly three years to low impedance electric and magnetic fields like those generated in earth by the ELF communications system. The principal finding of that earlier study was an enhanced growth rate in pubescent males exposed to ELF fields. The project reported here was designed to replicate the initial study and to evaluate the hypotheses developed concerning the cause of the growth effect. Thirty rhesus monkeys were exposed from 1 to 54 months of age to the same ELF fields used in the first study.

THE FINDINGS

Growth rates of exposed and control animals in this study were not significantly different from each other, although exposed males grew slightly faster than control males during puberty. None of the parameters measured showed statistically significant differences between the means of exposed and control subjects. The few parameters that showed statistically significant differences in the group versus time interaction were not correlated with one another and were judged to be isolated chance occurrences without physiological significance. The hypothesis regarding general anabolic effects of ELF exposure was rejected. Although the growth rate trends did agree qualitatively with the earlier study, the results of this second project failed to replicate the growth rate effect of the first study. The data from this project also failed to provide conclusive support for the hypothesis that stimulation of testosterone secretion was the cause of growth enhancement observed in the initial study. Throughout the study, the ELF exposed animals were in good health and showed no evidence of adverse physiologic effects from the exposure.

ACKNOWLEDGEMENTS

Opinions or conclusions expressed in this report are those of the authors and do not necessarily reflect the views or endorsement of the Navy Department. The project could not have been completed without the effective support of a large part of the Bioenvironmental Sciences Department staff. A special acknowledgement is extended to Mr. Dan Prettyman and Mr. Bob Upchurch for superb dedication and care in conducting the daily protocol for 4 1/2 years. We would also like to thank COL Tony David and CAPT Jack Bley for veterinary support, Mr. Bob Barrett and Mr. Chuck Mogenson for visual aids, Mrs. Anna Johnson for typing the report, and Dr. John Resko and Dr. David Hess for consulting support.

The animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, DHEW, NIH Publication No. 80-23, 1980, and The Animal Welfare Act of 1966, as amended.

INTRODUCTION

Since 1968, the Department of the Navy has repeatedly proposed construction of an extremely low frequency (ELF) system to communicate with submarines at operational depth and speed (4). Current methods to communicate with these submarines use very low frequency (VLF) signals and require the submarine to cruise slowly at shallow depths and trail a long antenna on or near the surface. An ELF system offers specific technical advantages, particularly in improving the submarine's capability to avoid detection. The Department of the Navy is currently completing an ELF communications system in Wisconsin and Michigan. In Wisconsin, a test facility constructed and operated since 1969 is being upgraded to operational status, and in Michigan a second transmitter and associated antenna system is being installed. These systems have been defined in detail in a Navy report (2). Throughout the history of the Navy's ELF proposals, considerable opposition from the general public has been encountered. This opposition has been strongest in the areas proposed as antenna sites, namely Wisconsin and Michigan. To evaluate the environmental and health effects of these fields, the Navy has sponsored numerous and varied research studies concurrently with the development of the project. The scientific literature primarily concerns ELF electromagnetic field exposure in air, often called high impedance fields. The fields produced by the Navy ELF system, however, are primarily generated in earth, i.e., low impedance fields. The Navy sponsored research has therefore been designed to provide information on fields in Earth. This research has been reviewed several times. The most thorough review of work relevant to ELF communications systems was reported by a Committee of the National Academy of Sciences (NAS) in 1977 (15), while a more recent review highlighted work conducted between 1977 and 1980 (8). The 1977 NAS report concluded that any risks from the ELF system to humans, animals, or the environment would be minimal. Nevertheless, controversy over such effects has continued to exist.

The NAS review included consideration of an interim report on a project at the Naval Aerospace Medical Research Laboratory (NAMRL) in which rhesus monkeys were being chronically exposed to ELF electric and magnetic fields designed to simulate those produced by the antenna. Later analysis of data from this project revealed that the exposed males had gained weight at a significantly faster rate than the control males. Since the committee did not have these data at the time of the report, the Navy asked the National Academy of Sciences to examine these observations in detail. A special panel was formed for this purpose. The panel concluded (16) that "The thoroughness and quality of the Pensacola [NAMRL] study were impressive," that there were "...no obvious errors in method or flaws in execution to account for the reported findings," that there was "...substantial evidence of a real effect of the Pensacola exposure conditions on the male animals," and that "Efforts should be made to define the effect further and to elicit its mechanism." The data reviewed by this special NAS committee covered the first 52 weeks of exposure of those animals. Following the recommendations of that panel, the exposure was subsequently extended. The final analysis of the project covered 2 years, 41 weeks of exposure. During the last year and a half of exposure, the rate of growth of the monkeys leveled off as somatic development was

completed. The size difference that developed during the first year of the exposure remained unchanged throughout this later period, but the variance of the groups (control and exposed) increased. When the full period of exposure was analyzed (2 yrs 41 wks), the growth rates of the control and exposed groups were not statistically different ($\alpha = 0.09$) from each other. Thus, the observed effect was limited to differences in growth rate of males during the pubertal growth period ($\alpha < 0.001$). A better understanding of the effect is necessary to assess the physiological significance of the finding, and to properly determine if exposure to such ELF fields is the true cause of the growth difference observed.

Since the growth effect was observed only in male subjects, it is logical to consider the possibility that the ELF exposure altered endocrine function. The study had not been designed to evaluate sex specific endocrine function, however. Efforts to evaluate parameters of reproductive endocrinology, e.g., testosterone or luteinizing hormone (LH) levels or sperm production, in the latter stages of the project were unsuccessful in revealing additional information about the sex-specific nature of the effect. It is possible that the basis for the growth rate difference was not related to sex, but only appeared to be so because of the difference in maturity at the start of the exposure. The males were just beginning puberty while the females were much closer to sexual maturity and maximum somatic development at the beginning of the exposure. Therefore, the females may have been less susceptible to a stimulus that affects growth rate. Growth rate also has a unique character among the many parameters that were measured, in that small differences consistently applied could accumulate to create a measurable difference after some time. Such a cumulative effect is unlikely to be seen in most, if not all, blood chemistry parameters because of the homeostatic controls on variation in these endpoints.

Three hypotheses were developed of possible mechanisms of the observed ELF enhancement of growth rate. These were (1) the ELF field has an effect on neuroendocrine function that results in stimulation of pituitary hormones, e.g., gonadotropins and somatotropin, which in turn, stimulate growth; (2) voltage gradients in the experimental apparatus can provide direct testicular stimulation that affects growth rate in males only; and (3) the ELF field has a generalized anabolic effect that increases the growth rate in males and females. To test these hypotheses, we decided to conduct a second experiment in which newborn rhesus monkeys of both sexes would be chronically exposed to the same conditions used in the first NAMRL chronic study. This would, first and foremost, test to see if the growth effect could be replicated. Secondly it would, by design, test the three hypotheses developed to explain the first study's findings. If the ELF field has a generalized anabolic effect, then growth differences should be most readily observed in young, rapidly growing animals and it should occur in both sexes. If the growth effect is due to direct testicular stimulation, then growth differences would be limited to males, would most likely occur after the onset of puberty, and might be indicated by elevated levels of circulating gonadal steroids. More complex possibilities exist if the ELF exposure affects neuroendocrine function. Such an effect might influence growth rate in both sexes. Growth differences might be observed only in the males, however, since anabolic steroids are produced in much

greater quantity by the male gonads than by the female gonads after the onset of puberty. Neuroendocrine effects might also result in a shift in the age at which the onset of puberty is seen in one or both sexes, or in alterations in the endocrine rhythms associated with sexual maturity in both males and females.

This report is the record of this second study of primates chronically exposed to ELF fields at NAMRL between April 1979 and June 1984. For details of the first study, hereafter referred to as ELF1, previous reports should be consulted (7, 9). In this study (ELF2) 30 rhesus monkeys (17 males, 13 females) were exposed for 22 hours/day from 1 to 54 months of age. A second group of 30 monkeys (17 males, 13 females) served as controls. Field conditions were identical to those of the first study. The biological endpoints examined were body measurements (11 parameters), gonadal endocrine function (4 hormones), hematology (11 parameters), and clinical examinations. No statistical differences in the mean values that were significant at or below the $\alpha = 0.05$ level were observed for any parameter in either sex. A trend in growth rate of exposed pubescent males was observed to be qualitatively similar to the results of the first study, but was not statistically significant.

MATERIALS AND METHODS

ANIMAL SELECTION AND PAIRING

Newborn rhesus monkeys (Macaca mulatta) of both sexes from the NAMRL rhesus breeding colony were used in this project. In the months preceding delivery, pregnant females from the colony were placed in individual standard wire primate cages in the vivarium. Each newborn monkey from these animals was carefully examined during the first month of life. If the animal was in good health, it was selected for use in the ELF project and was randomly designated as an experimental or a control animal. When the offspring was 1 month old, the mother and newborn were placed in the ELF exposure facility in a randomly predetermined location. The mother remained with the baby in the same cage until the baby was about 6 months old. At that time, the mother was removed from the ELF facility and returned to the colony. To reduce mortality and behavioral problems associated with growing up in social isolation, two infants were housed together in a single cage after weaning until the younger of the pair was 2 years old. The animals were paired as male-female except for four pairs of males (two pairs in each group, control and exposed). Pairing was also rigidly consistent by position between control and exposed groups. From 2 years of age until the end of the study, each animal was housed individually, with only visual contact with other monkeys. The data collection period was from 1 to 54 months of age. Monkeys were removed from the facility within a few months after they were 54 months old, and, with few exceptions, all of the ELF2 project animals have now been transferred to other laboratories for subsequent use.

As noted above, entry into the project was randomized using a scheme that anticipated the later pairing of young animals after weaning. Blocks of four positions (2 in each group) were lettered successively. A random permutation of the 15 blocks of 4 positions was determined (3). The blocks

were filled in the order of the random number assigned to the block and the positions were filled in the order of their assigned number within the block (see Table I). A random sequence of treated and control subjects was determined separately for males and females. Sixty-eight envelopes designated male 1 to male 34 and female 1 to female 34 were prepared. Each envelope was filled with a designation of red or blue. Each assignment was made on a random basis and a random block sequence was used to ensure that there was uniformity in the ages of the two groups of animals. When an animal was ready to enter the experiment, the envelope designating its entry number and sex was opened and its designation as red or blue was determined. The animal was then placed in the next available cage of that color designation according to the procedure outlined above. Positions were held open until an animal of the same sex was obtained for each group for the same position.

Consideration was given to blocking based on male parentage to prevent offspring of a given male from being assigned disproportionately to either experimental or control group. The following procedure was adopted. When the difference in experimental or control assignments from a given male reached two, the method for assigning the next animal from that male was slightly modified. The normal procedure was followed if the outcome did not increase the disparity, but if the outcome would have increased the difference, then the final decision was deferred to another file of sealed envelopes that had been prepared for the contingency. Each of these envelopes contained one of two instructions "change" or "no change." These envelopes were randomly prepared with a 70 percent probability that the instruction would be "change" and a 30 percent probability that the instruction would be "no change." These two instructions simply meant that the assignment specified by the normal procedure should either be changed or allowed to stand. Obviously the disparity could have been increased, but the probability was greater that it would be reduced. The actual distribution of offspring in the project with respect to fathers is shown in Table II.

The decision to use a total of 60 animals (30 exposed, 30 control) was, for this study, based on the facilities available that were built for the first NAMRL primate study (ELF1). Although the statistical power and level of confidence of an experimental design can be theoretically calculated for any sample size, such calculations could not be made prior to the NAMRL ELF1 project because the variances of the parameters to be measured were not known. Due to these limitations, the sample size of 30 per group was selected for ELF1 as the maximum number of subjects that could be exposed with the logistical constraints in effect (building size and budget). It was not originally planned to evaluate the data with separate groups by sex of the animals. The decision to do that later, and the subsequent discovery of gender-specific effects, reduced the effective sample size by approximately half. This reduction in sample size clearly reduced statistical power for the projects and increased the possibility of either a Type I or Type II error (11) in the interpretation of the results. These statistical limitations are recognized by the authors, even though an alternative approach to overcome them was not feasible.

TABLE I

Randomized Scheme for Placement of Animals in ELF Facility

Block Designation	Order of Filling Block	Order of Filling Position w/in Block
A	6	1 2
B	14	1 2
C	10	2 1
D	2	2 1
E	8	2 1
F	15	2 1
G	11	1 2
H	3	1 2
I	5	1 2
J	1	1 2
K	7	1 2
L	13	2 1
M	9	2 1
N	4	2 1
O	12	2 1

TABLE II

FATHER - OFFSPRING DISTRIBUTION

FATHER	MALES		FEMALES		TOTAL
	CONTROL	EXPOSED	CONTROL	EXPOSED	
540A	1	0	2	2	5
AR4	2	0	1	0	3
632B	0	2	0	0	2
472B	1	1	1	1	4
688B	0	1	0	0	1
AP6	0	1	0	0	1
370A	1	1	0	1	3
209B	3	1	3	2	9
942B	1	1	2	1	5
556A	2	0	1	1	4
986A	2	1	1	0	4
958B	0	2	0	0	2
24C	2	2	0	1	5
586B	2	3	1	1	7
566A	0	1	1	1	3
626A	0	0	0	1	1
552A	0	0	0	1	1

Animal chambers

The apparatus and facilities used in this study were essentially the same as those used in the first NAMRL chronic exposure study with rhesus monkeys. This was true of the animal chambers as well, although major parts of these chambers were replaced with new ones built for this study. The chamber was built of Plexiglas except for the electrical conducting components, and had three sections. The bottom included the grid of stainless steel bars that was the floor of the chamber for the animal, the resistors through which the current passed, and a reservoir beneath the

grid floor to hold urine and feces. The main body of the chamber was rectangular, providing a confinement area of 0.6 x 0.6 x 0.76 m for the animal. It contained a food hopper and holder for a water bottle near the top (see Figure 1). The chamber was closed by a single sheet of Plexiglas that slid into grooves at the top of the main body of the chamber. The top was perforated with numerous holes through which room air was drawn into the chamber by an exhaust fan that pulled the air out at the bottom and exhausted it to the atmosphere outside the building.

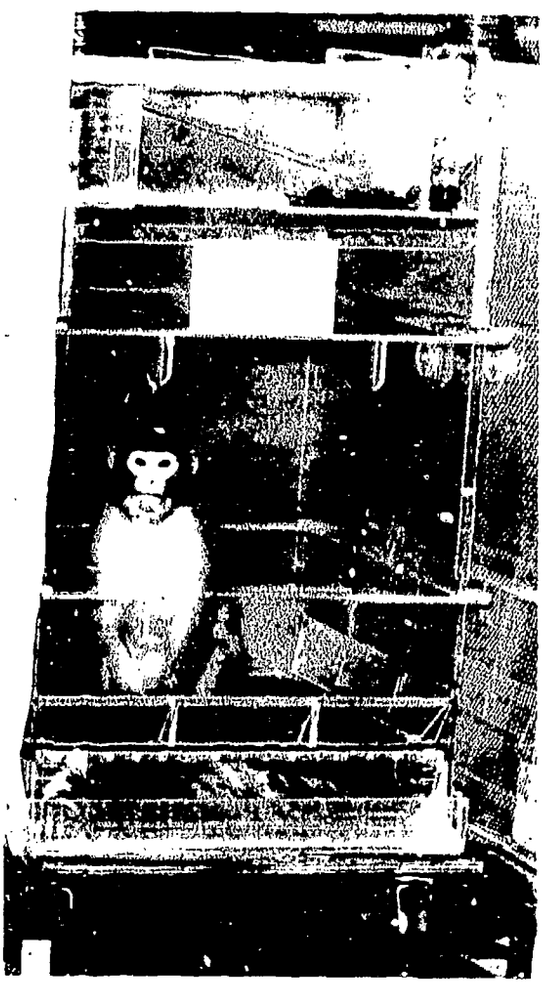


Figure 1

Animals lived in Plexiglas cages made of three sections. The bottom section held current conducting metal bars that provided the floor of the chamber. The sliding top was locked in place by the water bottle and contained ventilation holes. The screens on either side of the chamber served to produce a uniform electric field.

EXPOSURE FACILITY

The ELF exposure facility was contained in a building totally dedicated to this project. The exposure system was designed in 1974 to simulate the electric and magnetic fields that would be produced by the proposed Navy submarine communications antenna. Since then, the proposed antenna design has been changed (4). One major change has been that the older design called for a buried cable antenna, while the antenna under construction will utilize primarily above ground cable. As a result of the design changes, the fields used in this study simulate only in part those fields associated with currents in the Department of the Navy's ELF communications system transmitting antennas. The building consisted of a central core area and two wings. The core area contained offices, cage washing facilities, a veterinary examination room, a food preparation area, field generating equipment, and other general purpose areas. The two wings were symmetrical and were designed so that either one could be used to expose experimental animals. Each wing had identical field generating equipment. When the experimental animals were in position in the north wing, the control animals were in corresponding positions in the south wing, but only the north wing field generating system was energized. There was no evidence that the two wings differed environmentally in any appreciable way, e.g., environmental control equipment, data transducers, noise level, vibrations, etc. Nevertheless, this system allowed the two groups of monkeys to be transposed each week, thereby balancing any subtle differences that may have existed. The Plexiglas chambers housing the 30 animals in each group were situated in a single row in the center of the wing. This row of cages was located directly beneath a row of full-spectrum fluorescent lights. The light cycle was 12:12 hours light:dark throughout the project. The transition between light and darkness was abrupt. No extraneous or low level light was permitted during the dark cycle. The windows into the building core area were covered with black curtains. Thus, these animals lived from birth in an environment controlled for light and temperature. The temperature of both wings was controlled by the same equipment at 23 $\pm$ 2 °C (range). These conditions were the same in all animal enclosures, and only minor (< 2 °C) seasonal variations existed.

The electric field generating system was an integral part of the animal chamber, as illustrated in Figure 2. The bars that formed the walking surface for the animal were constructed of 1.27-cm square stainless steel bar stock. These lay in slots spaced 3.81 cm between centerlines and rested on thin stainless steel strips 3.5 cm long that bridged the floor of each slot. These strips were connected by 3900-ohm resistors. The end resistors were connected to a stainless steel strip that passed down into the trough and terminated on the outer edge of the feces tray. A current source was connected via these terminations to the network of resistors and an electric field gradient of 0.76 volts was generated between adjacent bars. Thus, the electric field gradient along the walking surface of the cage was 20 V/m. The current source for this electric field simulator was driven by an amplifier with an input from the same modulator used for the magnetic field generator. Wire screens indicated in Figure 2 (and shown in Figure 1) were placed on each side of the chamber and were connected to the same voltage source that energized the resistor network. These screens

created a uniform horizontal electric field similar in orientation to that near the ELF antenna.

The electric field distribution in the animal chambers was measured with an Illinois Institute of Technology Research Institute (IITRI) high impedance electric field probe in conjunction with a Hewlett Packard (HP) 3581 Wave Analyzer. At four elevations in the chambers, measurements were taken near the corners and in the center as shown in Table III. The electric field screens would be on the right and left. These particular values were recorded at position 30 in the south wing. They are, however, typical of values recorded at other positions, with an average electric field strength of 20 V/m in air. That level exceeds the nominal values of electric fields at the Wisconsin Transmitting Facility and the Michigan Transmitting Facility by factors of approximately 150 and 300, respectively. The electric fields associated with transmitting antenna voltage, which are vertically oriented relative to the Earth's surface and exist only in air, were not simulated in this experiment.

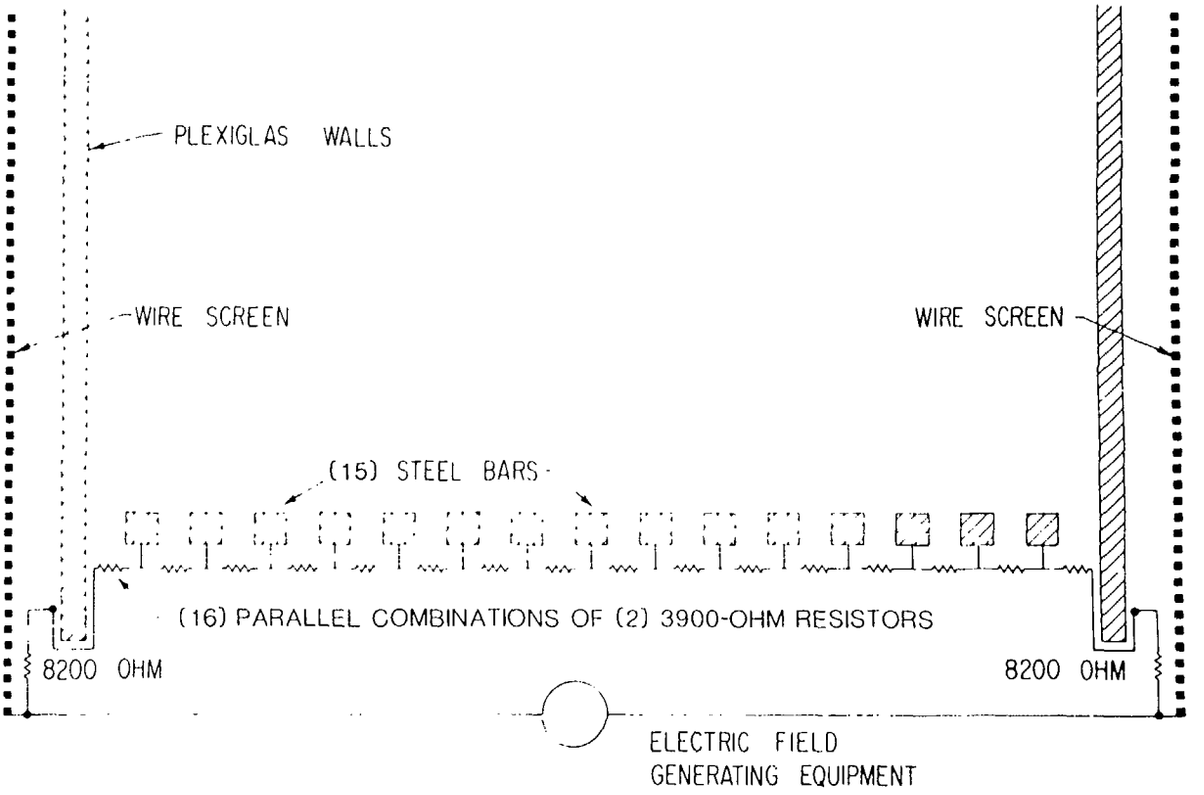


Figure 2

Schematic of the electric field generating system. Identical networks of resistors were at both ends of the bars so that the net resistance between bars was actually formed by two 3900-ohm resistors in parallel.

The magnetic field generating system was intended to simulate the magnetic field associated with an ELF communications system. Those currents produce magnetic fields that are horizontally oriented near the Earth's surface close to the antennas. The field direction was horizontal in the north-south direction. The coil system consisted of three parallel wire bundles 26 m long. These bundles were laid in concrete trenches 1.1 m deep and 1.1 m wide. The bundles were spaced at 3 m and connected at each end such that the entire system consisted of a single continuous copper wire cable wound in the form of two adjacent rectangles with a long common side. Eighty-six turns were in the center trench, 48 turns in the outer trench and 38 turns in the inner trench. The reinforcing steel bars in the concrete were broken at intervals to prevent inductive generation of a secondary current loop in the steel bars. The trenches were covered with plywood, and a row of 30 animal chambers was placed approximately 1.1 m directly above the center coil. The system generated a field of 0.2 mT (2 gauss) in the chambers and simultaneously generated a null field in the control chambers placed along a parallel line 25.8 m from the experimental chambers. The control chambers were placed above a coil system identical to that which exposed the experimental animals. These identical coil systems could not be energized at the same time. The modulator and amplifier were switched to the other coil when the animals were transposed. The group first designated as experimentals always retained that status. They were simply exposed by a different coil system on the opposite side of the building.

The magnetic field distribution in the animal chambers was measured with an IITRI magnetic field probe in conjunction with a Hewlett Packard 3581 Wave Analyzer. At three elevations, measurements were taken near the corners and in the center as shown in Table III. Adjacent chambers would be on the right and left and the magnetic field vector would be in the horizontal plane and perpendicular to the row of animals. These particular values were recorded at position 30 in the south wing. They were typical of values recorded at other positions and for the other field generating system. These values exceed the magnetic flux densities produced at the Wisconsin Transmitter Facility by a factor of 33, and those that will be produced at the Michigan Transmitter Facility by a factor of 66.

The exposure system was driven by a single electric generating source located in the center of the building midway between the two wings. The shortest distance to the cage line in either wing was exactly the same, 12.9 meters. At pseudorandom intervals, the oscillator shifted between 72 Hz and 80 Hz. At the time of the shift, the coil system was instantaneously tuned to the correct frequency. This automatic tuning was accomplished by solid state switching that changed the value of total capacitance in resonance with the coil system. The frequency spectrum of the signal source was measured by a Navy contractor (IITRI) and determined to be a good simulation of the actual ELF antenna situation.

The data in Table III was taken prior to the start-up of ELF1 in the summer of 1975. A magnetic field probe located beneath position 15 in each wing provided on-line verification of the presence of the magnetic field throughout the study. The current flowing to the electric field generating system and to the coil system were also continuously monitored. In

TABLE III

FIELD DISTRIBUTION

	HEIGHT ABOVE WALKING SURFACE	POSITION				
		Left Front Corner	Right Front Corner	Center	Left Rear Corner	Right Rear Corner
Electric Field Distribution (V/m)	69 cm	6.350	12.220	14.080	8.410	9.010
	52 cm	22.110	28.350	17.000	24.530	26.830
	42 cm	24.290	25.620	19.650	27.990	23.460
	10 cm	27.930	22.440	19.110	28.450	20.540
Magnetic Field Distribution (mT)	69 cm	0.118	0.117	0.109	0.110	0.108
	42 cm	0.147	0.144	0.146	0.144	0.141
	10 cm	0.203	0.189	0.194	0.197	0.197

addition, the electrical integrity of each individual system (cage and associated screens and resistors - see Figure 2) was verified daily. These checks ensured that the fields were maintained as described in Table III, even though periodic verification of actual fields in the chambers was not done.

EXPERIMENTAL PROTOCOL

The basic experimental protocol was for 22 hours of uninterrupted exposure per day, 7 days per week. The fields were turned off at the time the lights came on each morning (0800) and remained off for 2 hours while data collection, feeding, watering, cleaning, and other routine operations were completed. As described above, the animals were placed into this protocol at 1 month of age. They continued in the experiment without interruption until they were 54 months old. Data collection was based on a 12-week cycle that began at birth.

A radiograph of the left fore and hind limbs was taken every 12 weeks to document bone growth. A thorough physical examination of each animal was conducted every 6 weeks. Weight and body measurements (or biometrics) were made every 2 weeks until 22 months of age, and every 3 weeks from 22 to 54 months of age.

Blood sampling was done on one protocol for both sexes from 6 weeks to 18 months of age, and on different protocols for each sex thereafter. In the initial protocol, three samples were drawn at 1-hour intervals on the morning of a collection day for a given animal. Blood collection days occurred on the first and third week of each 6-week cycle. Thus, an average of one sample per week was drawn from each monkey. These samples were drawn by heel-stick capillary puncture until the monkey was 6 months old, and by femoral venipuncture after that. The blood volume collected per sample was 0.5 ml for animals less than 3 months old, 0.75 ml from 3 to 6 months of age, 1.5 ml from 6 to 12 months, 2.0 ml from 12 to 22 months, and 3.0 ml after 22 months of age. At 18 months of age, blood sample collection was altered for females to provide a protocol designed to document the onset of puberty and the sexual maturation process. For males, the initial protocol continued until age 22 months. After 22 months of age, three samples were collected on a particular day, Wednesday, from each male once every 3 weeks. These three samples were drawn at 0900, 1000, and 2200 hours, i.e., 1 and 2 hours after the lights came on in the morning, and 2 hours after the lights went off at night. To accomplish the night draws, the exposure and light cycle were interrupted for about 1 hour each Wednesday night. One-third of the males had blood drawn each week, with an equal number of animals from each group involved. For females, a single sample was drawn on a collection day twice per week. One half of the females were in a Monday - Thursday collection group, while the other half were in a Tuesday - Friday collection group.

The two groups of animals, control and exposed, were completely transposed from one wing of the facility to the other each Wednesday. Each animal retained the same position in the row of animals on either side, with the same neighbors in the line. Each monkey was transferred to a clean cage once each week. In order to evenly distribute the work load,

one-fifth of the animals (six) from each group received clean cages each normal working day, i.e., six on Monday, six on Tuesday, etc. However, for a particular monkey, the cage change always occurred on the same day of the week. Each monkey was provided a measured amount of food (Wayne Monkey Diet 8663, Allied Mills, Inc., Chicago, IL) and water (900 ml) each day. The daily ration of food was increased as the monkeys grew, from about 150 g/day for a recently weaned animal to about 250 g/day for a 4-year old. Many of the animals did not consume all of the food offered. Uneaten bisquits were frequently observed in the waste tray of the chamber.

RADIOGRAPHIC PROCEDURE

Periodic radiographs of the monkeys were taken of the extremities on the left side, showing the entire limb distal to the shoulder and hip joints, including the proximal epiphyses of the humerus and femur. These techniques involved minimal radiation exposure, requiring energies of about 40 to 50 kV at 400 mA for 1/120 second. Measured exposures for these machine parameters ranged from 0.0022 to 0.0051 rem for estimated skin dose in a single exposure.

BIOMETRIC PROCEDURES

Techniques for making the body measurements remained the same throughout the study with one exception. Prior to 22 months of age, the measurements were made on alert, unanesthetized animals. After 22 months, the animals were anesthetized with a 10 mg/kg ketamine hydrochloride intramuscular injection, (Park Davis, Morris Plains, NJ) prior to measurement, in order to avoid injury to either the animal or the technician from the struggle of restraint.

In general, the techniques used in this study were similar to those described by Schultz (14). Our noninvasive measurements were of an anatomical region rather than a specific bone. Measurements were made of head width, head length, shoulder width, hip width, upper leg length, knee height, lower arm length and chest circumference as well as body mass. Calipers were used for all biometric measurements with the exception of sitting height and chest circumference. Head width, upper leg length, knee height, and lower arm length measurements were taken using a sliding compass with adjustable arms. An outside caliper was used to measure head length, shoulder width, and hip width. The ability to replicate biometric measurements required a "touch" for how tightly the calipers fit over the measured area. The calipers had to be tight enough to move the skin over the underlying tissue, but not tight enough to bind. Special care was taken to ensure proper positioning of the animals and correct orientation of the instrument to the area measured.

Head width was defined as the widest part of the head above the ears in a direction perpendicular to the midsaggital plane. The sliding arm of the caliper was adjusted to measure the distance between the protrusions of the temporal lines at the juncture of the temporal and parietal bones. Head length is the distance at the midsaggital plane from the fleshy area between the superciliary arches to the lower parietal area just above the inion. These measurements are schematically illustrated in Figure 3.

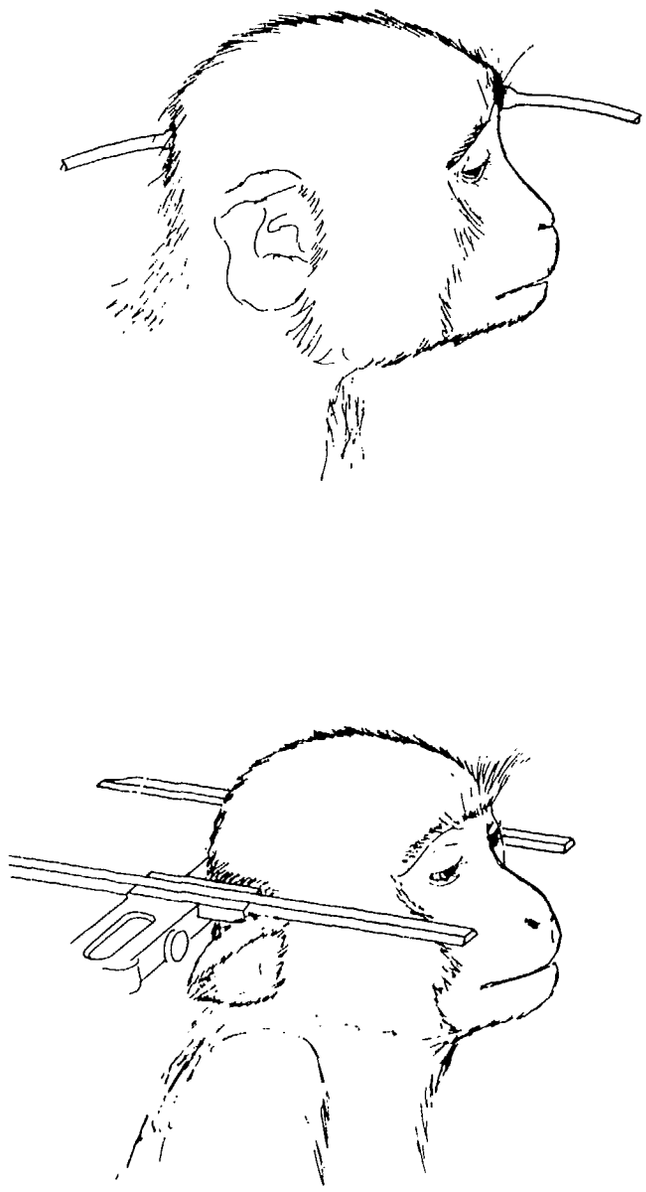


Figure 3

Sketch of the measurement technique for head length and head width.

Shoulder width is the widest distance between the tubercullum majors. To ensure reproducible results, the animal's arms were held together at the elbows behind its back. It was also necessary to palpate the bone to be sure that the measuring points were over the bony surface and not on the surrounding musculature.

Hip width was measured with the ends of the open calipers on the two points of the greater trochanter. It was observed that varying the positions of the legs caused variations in this measurement. To obtain a consistent result, all measurements were taken with the animal in a standing position with the legs held together at the knees.

The measurements of the extremities were designed to approximate, with a noninvasive technique, the lengths of the major long bones in those regions. The upper leg is the distance from the top of the greater trochanter to the patella. It was measured with the leg in a sitting position, i.e., bent at the hip and knee. The stationary arm of a sliding compass was placed against the top of the greater trochanter, and the sliding arm of the compass was adjusted to fit tightly over the patella with the caliper parallel to the axis of the femur. Knee height was measured with the knee bent and the foot perpendicular to the axis of the lower leg, as illustrated in Figure 4. One arm of the sliding compass was placed over the medial femoral condyle and the other arm was situated over the heel. The lower arm dimension is the distance from the olecranon to the heel of the hand. The arm was bent at the elbow and the hand was flexed back for this measurement.

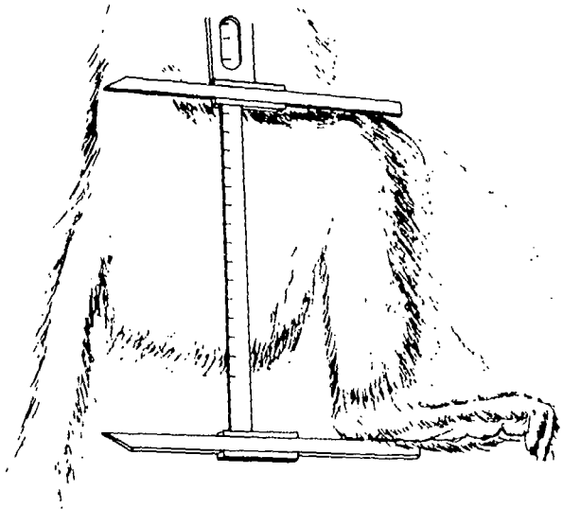


Figure 4

Sketch of the measurement technique for knee height.

The apparatus shown in Figure 5 was used to measure sitting height. The animal was placed on its back with the rump pressed against the stationary stop. The animal was held perpendicular to the stationary stop. The sliding piece was pressed against the head and the distance between the stationary stop and the sliding piece was measured. This biometric parameter proved to be the most difficult to obtain reproducible results. The following conditions were essential: (1) the spine had to be positioned perpendicular to the stationary stop, (2) the head had to be facing straight up with the neck neither stretched or compressed, and (3) the animal had to be relaxed if not anesthetized. Due to the inherent inaccuracies of this measurement, the average of five repetitions was used as the measurement of record.

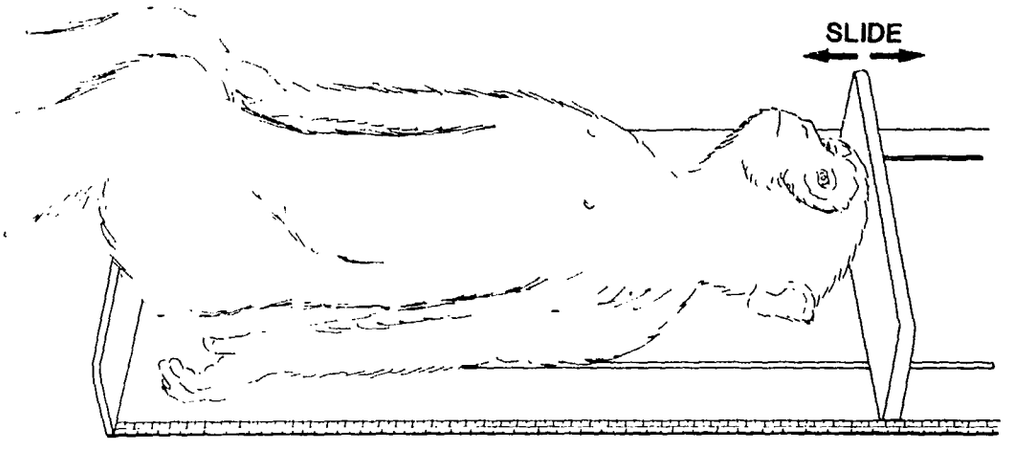


Figure 5

Sketch of the measurement technique for sitting height.

During the last 6 months of the study, chest circumference and testicle size were also measured in all males of both groups. Chest circumference was measured with a tape measure with the animal in an upright, sitting posture. The measurement was made at the level of the papillae mammae. While the animal was anesthetized for biometric measurements, length and width of the testicles were measured with sliding calipers. Using the length and width as major and minor axes of an ellipsoid, respectively, the volume of the glands was calculated.

BLOOD SAMPLE ANALYSIS

The primary purpose for collecting blood samples was to determine steroid hormone levels in the circulating blood. It was planned that

pituitary hormones, particularly luteinizing hormone (LH), would be measured only if steroid hormone levels showed a difference between experimental groups. Each blood sample was identified with a unique control number that had no relationship to the experimental status of the animal. At the time the blood was collected, a label containing that control number was placed on the collection tube. When the serum (or plasma) was separated after centrifugation, that label with the control number was transferred to the storage vial containing the serum. For assay purposes, only the control number was available to the technician performing the work. Thus, the analyses of blood samples were conducted without any knowledge of the group or animal from which the sample was taken. Data from these assays were then entered into the computer by control number and electronically decoded to reestablish the identification with the correct animal. Blood collected by femoral venipuncture (after age 6 mos.) was allowed to clot in test tubes at 4 to 10 ^{o}C for 2 to 8 hours. The blood was then centrifuged and the serum was removed and stored at -85 ^{o}C until it was assayed. Capillary puncture samples were collected in heparinized tubes, centrifuged promptly, and the plasma was frozen (-85 ^{o}C) for later analysis. Occasional samples drawn by venipuncture were collected in tubes containing ethylenediaminetetraacetate (EDTA) as an anticoagulant so that hematological analysis could be performed. Under the prepubertal protocol in which three morning samples were drawn on a given day from each animal, the first sample was collected in this manner for hematology. After the protocol changed during the peripubertal period, this routine was continued for the males, with the first morning sample being processed for hematology. For the females from which samples were drawn twice per week, the first sample drawn each month was processed for both hematology and steriod analysis. Samples collected for hematology were left on a slowly oscillating mixer until the hematological measures were completed within 2 to 4 hours. These samples were then centrifuged, and the plasma was separated and stored frozen for subsequent steroid analyses.

The steroid hormones measured were estradiol-17 (E2), progesterone (P), testosterone (T), dihydrotestosterone (DHT), and 4-androstenedione. Not all of these hormones were measured in every sample analyzed. Not all of the samples collected were analyzed for hormone concentration. All of the samples collected from males between ages 28 and 54 months were analyzed. The analysis of these peripubertal male samples always included measurement of T and DHT, and sometimes included measurement of E2 and androstenedione as well. Samples collected from females between 24 and 50 months were always analyzed for E2 and P. Some female samples were analyzed for T and DHT as well. Only one of the two weekly female samples was analyzed at the early part of this age range. After increased P levels indicative of corpus luteal activity were noted in a specific monkey, every subsequent sample from that female monkey from the age of corpus luteal activity onset up to 51 months of age was analyzed.

The steroid hormones were determined by a combination of extraction, chromatography, and radioimmunoassay (RIA) procedures according to the techniques of Resko, et al. (12, 13). Serum (or plasma) was extracted with freshly distilled ether and the organic phase was transferred and dried in preparation for chromatography. Liquid chromatography was performed on

Sephadex LH-20 columns in a solvent mixture of hexane, benzene, and methanol. Eluent fractions from the columns were automatically collected with a model MCC-1 multiple column fraction collector (Hollyhills Biologicals, Inc., Hillsboro, OR). The samples were first chromatographed on columns containing 1.0 g of Sephadex LH-20 in a hexane: benzene: methanol mixture of 62:20:13 ratio to separate E2 from neutral steroids and from the other estrogens (13). The eluent fraction containing the neutral steroids was then chromatographed on a column containing 2.5 g Sephadex LH-20 in a mixture ratio of 85:15:5 hexane:benezene:methanol to separate the neutral steroids progesterone, Δ4-androstenedione, T, and DHT (12). Each steroid was then quantitatively measured by RIA of the appropriate chromatography eluent fraction. Commercially available antibodies (Hollyhill Biologicals, Inc., Hillsboro, OR) were used to perform RIAs for E2, P, T, and DHT. Dr. Resko kindly provided the antisera used to measure Δ4-androstenedione. Antibody bound and unbound hormone were separated using a dextran-coated-charcoal suspension. Tritium-labeled hormones were purchased from Amersham, Inc. (Arlington Heights, IL).

The analysis of male serum (or plasma) samples for steroid hormones was conducted at NAMRL. The analysis of female samples was performed under contract by the Radioimmunoassay Lab of the Oregon Regional Primate Research Center (ORPRC), Beaverton, OR. Samples analyzed in Oregon were shipped frozen by air freight from NAMRL to the contractor. Included in each shipment were quality control samples of pooled monkey serum containing low or high levels of the steroids of interest. These pooled serum samples were not identified to the contractor, but were randomly included in each shipment. These pooled samples were also taken from the same pool used at NAMRL for quality control in steroid assay procedures. A comparison of results on the pooled serum values from the contractor and NAMRL is shown in Table IV. The pooled quality control serum was assayed as two or more independent samples in each batch. The mean and variance of these replicates are referred to as "within-assay" values. The "between-assay" values shown in Table IV are the overall means and variances of the replicate means of all batches assayed. The coefficient of variation shown here was determined as the standard deviation divided by the mean and expressed as a percent. The data were arbitrarily grouped by years to provide periodic comparisons. The number of assay batches was more than 30 in each year. Coefficients of variation of 20 percent or less are generally considered satisfactory (1). This level of proficiency was achieved in nearly every case by both the contractor and NAMRL. With the exception of the E-2 level in the high pool control serum (1983), mean levels for each hormone analyzed by both NAMRL and the contractor were in excellent agreement.

Hematological analyses of the blood samples included a complete blood count (CBC) with differential. The parameters determined were erythrocyte or red blood cell (RBC) count, hemoglobin, hematocrit, mean corpuscular volume (MCV), leukocyte or white blood cell (WBC) count, and differential counts of white blood cells, specifically neutrophils, lymphocytes, monocytes, eosinophils, basophils, and bands. Coulter Model ZBI or ZM particle counters (Coulter Electronics, Inc., Hialeah, FL) were used to determine WBC, RBC, and MCV. Hematocrit was automatically calculated from these parameters by a Coulter Model MHR computer accessory, and hemoglobin

TABLE IV

HORMONE ASSAY QUALITY CONTROL[a]

		1981 CONTROLS[b]	1982 CONTROLS	1983 CONTROLS	1984 CONTROLS
		MEAN ± SD (%CV)	MEAN ± SD (%CV)	MEAN ± SD (%CV)	MEAN ± SD (%CV)
LOW CONTROLS					
E-2	NAMRL	74 ± 16 (22)	48 ± 8 (17)	96 ± 14 (14)	
	CONTRACT			80 ± 17 (21)	11 ± 2 (16)
P	NAMRL			1404 ± 175 (12)	2050 ± 201 (10)
	CONTRACT				
T	NAMRL	458 ± 96 (21)	154 ± 31 (20)	275 ± 56 (20)	317 ± 31 (10)
	CONTRACT			310 ± 35 (10)	
DHT	NAMRL	647 ± 10 (15)	351 ± 77 (22)	231 ± 63 (27)	428 ± 88 (21)
	CONTRACT			248 ± 30 (12)	
HIGH CONTROLS					
E-2	NAMRL	372 ± 69 (18)	221 ± 77 (35)	533 ± 66 (12)	
	CONTRACT			362 ± 57 (16)	158 ± 10 (6)
P	NAMRL			3138 ± 452 (14)	3284 ± 43 (1)
	CONTRACT				
T	NAMRL	899 ± 151 (17)	522 ± 90 (17)	1791 ± 200 (11)	1812 ± 194 (11)
	CONTRACT			1780 ± 129 (7)	
DHT	NAMRL	1555 ± 160 (10)	1152 ± 288 (25)	2101 ± 366 (17)	2654 ± 170 (6)
	CONTRACT			1928 ± 234 (12)	

[a] Values shown are the between-assay means, standard deviations, and coefficients of variation percentages (%CV). Within-assay variability was consistently less than between-assay variability.

[b] Control samples were taken from large volume pooled monkey serum batches that had been spiked with hormones to produce desired levels for "high" controls. Low controls were direct assay of the pooled serum without any added hormone.

concentration was determined with a Coulter hemoglobinometer. The differential counts were the percentages noted in counting 100 leukocytes on a blood smear stained with Wright's stain and observed under a microscope oil immersion objective. The primary purpose in performing the hematology work was to provide the veterinarians with an additional assessment of the clinical health of the animals.

VETERINARY CLINICAL EXAMINATION

As noted earlier, each animal received a thorough physical examination by a veterinarian every 6 weeks. The experimental and the control monkeys from paired cage positions were always given exams on the same day. The veterinarian's examination included the following: observation of ocular motility, direct and indirect pupillary reflexes, facial muscle tone, locomotor and proprioceptor activity, disposition and demeanor; visual and manual examination of head, face, scalp, neck, mouth, teeth, throat, extremities, skin, haircoat, superficial spine, and perineal region; direct ophthalmoscopy; otoscopic visualization of external auditory canal and nares; palpation of abdomen, superficial lymph nodes, femoral pulse, and inguinal canals; and auscultation of heart and lungs. Tooth eruption and development of secondary sexual characteristics were noted.

Under the direction of the veterinarian, rectal swabs were taken for bacteriologic examination on a less frequent, but periodic basis. Intradermal tests for tuberculosis were conducted every 6 months. The veterinarian and his technical staff also performed the following tasks daily: visually examined every monkey and chamber; inspected sanitary conditions of the animal areas, the examination room, and the cage washing areas; and reviewed records and minor health problems of any animals requiring special observation. Beginning at 18 months, the perineal region of each female was examined twice per week, at the time of blood collection. The presence of dried or fresh blood on the perineal area was considered to be evidence of menstruation.

STATISTICAL ANALYSIS PROCEDURE

Data with a normal distribution or with the capability of being transformed to a normal distribution were statistically analyzed with a two-factor analysis of variance (ANOVA) by using repeated measurements on one of the factors (18). These methods have been extensively described in a previous report (9). An unweighted means analysis was employed to compensate for unequal cell frequencies encountered because of missing data. In the statistical analysis of these data, two sources of variation were of direct interest in making decisions about the effect of the exposure level upon the animal groups. These are the exposure level (control or exposed) and the interaction between exposure level and exposure duration. The F-ratio obtained from the exposure level factor can be used to make decisions about the differences between the means of the two groups of animals. The interaction can be used to determine whether the two groups responded differently to the exposure duration factor (a group versus time interaction).

The exposure duration factor, however, does not have a direct bearing upon the question of whether differences between the two groups were caused by the field treatment. The time series records of animals are subject to a number of factors other than the exposure level factor. For example, seasonal variations of blood parameters constitute an additional factor common to both groups, but not related to the field exposure.

The exposure level factor assumes that there were no initial differences between the two groups of animals. If the assumption is false, the effects of the field level are said to be confounded with differences between groups. A significant F-ratio for the interaction would indicate a difference in the simple effects of the two exposure level factors. Thus, these differences occurred between the two groups as a function of time, but such differences are not indicative of clear trends in the two groups.

The Mann-Whitney U-test (5) was applied to those parameters that were not normally distributed and could not be readily transformed to normal distributions. The Mann-Whitney U-test is a rank test that can be used to determine the level of significance.

For the growth parameters (mass and biometrics) and hematological indices, data from the 53-month period of study were condensed by averaging measurements over 6-week intervals. These 6-week average values were then used in the statistical analysis and subsequent graphing of these data. Two derived parameters of growth were added in the condensed data format to evaluate the relative amount and rate of growth. These derivations of body mass data were body mass ratio, which relates subsequent mass to the initial body mass at 1 month of age, and growth rate, defined as the gain in body mass per 6-week period.

Data from the hormone measurements were not condensed for analysis because of the rhythmic pattern in circulating levels. Some specific analysis of these rhythms was done on data from individual subjects. In addition, the mean data for the two groups was tested by ANOVA, with the limitation that only the time intervals of 106 to 156 and 156 to 234 weeks of age could be tested (separately) because of limitations in the storage capacity of the computer used.

A difference between treated and control subject data was considered to be statistically significant when the alpha level was ≤ 0.05. Differences between the two groups for which alpha levels were > 0.05 were not considered to be statistically significant. Statistically significant effects have been noted whenever the alpha level met the above criteria for one or more of the factors of the analysis.

RESULTS

The growth curves for body mass, the two derived parameters of growth in mass, and the eight biometric measurements are shown in Figures 6 to 16, for males and females. Note that the abscissa is weeks of age, which is directly related to weeks of exposure. There was about 9 months difference in age between the oldest and youngest males (5 months for

females), so these data are not synchronized in chronological time. Thus, data for individual animals that were averaged to produce a particular point on a graph (e.g., weeks of age = 200) were accumulated over a 9-month period of time for males, and a 5-month period of time for females. This means that an unusual event that might cause an artifact, such as equipment failure on a given day, would affect only a few animals contributing to a specific data point. No statistical differences were found (Table V) between exposed and control groups for any of these growth parameters, with the exception of head width in males, and upper leg length in females. The alpha level ($< .001$) for the group versus time interaction term for male head width is a result of some divergence in the curves of the two groups (Figure 12) over the latter months of the experiment. The cause or significance of this difference is unclear since no other parameter of growth shows a similar effect over the same time period. In females, the significant interaction (group versus time, $\alpha < 0.05$) for upper leg length is also an isolated trend of slight magnitude that is uncorroborated by other parameters. The gradual transposition and divergence of the male growth curves (Figure 6) between weeks 120 and 210 indicates that the exposed males were growing slightly faster than the control males during this period. The 2 groups of females showed differences in mass that were as large at times (Figure 6) as the males showed at week 210, but the relative rates of growth of the 2 groups of females fluctuated up and down throughout the experiment in contrast to the more uniform trend of the males. This trend toward faster growth in exposed males is more readily apparent in the derived parameters, particularly in body mass ratio (Figure 7). The absence of any consistent difference in growth rates of females is also more readily seen in body mass ratio. Note also that a pubertal growth spurt is clearly evident for both groups of males beginning at about week 180 (Figure 6). A similar growth spurt in females was not observed (Figure 6).

Chest circumference showed no appreciable difference between male groups over the last 6 months of the study. At 48 months of age, the chest circumference measured 344.1 ± 3.2 and 346.9 ± 3.5 cm (mean $\pm$ SE) for the exposed and control males, respectively. At 54 months of age, chest circumference was 364.8 ± 4.2 and 365.7 ± 3.8 cm for exposed and control males, respectively.

Testicle measurements showed a rather high degree of variability, both in individual monkeys and within groups. This was a reflection of the difficulty of making accurate measurement *in situ*. Little change in testicular volume was noted in individual animals during the 6-month period of measurement (48 to 54 months of age). The average volume of the left testicle was not significantly different between exposed and control groups. This volume was 21.4 ± 2.9 and 17.0 ± 2.2 cm^3 (mean $\pm$ SE), respectively for exposed and control males.

Menstruation in females and an evening elevation in testosterone level were taken as indicators of the onset of puberty in females and males, respectively. The first ovulation, evidenced by elevated progesterone (P) values, is also a clear and significant event in sexual development. Slight differences in the mean age of the occurrence of these events (Table VI) between exposed and control animals of either sex were not

TABLE V

ELF2 STATISTICAL SUMMARY

PARAMETER	SEX	TRANSFORMATION CODE*	DIFFERENCES BETWEEN MEAN (ANOVA)			GROUP VS TIME INTERACTION (ANOVA)			RANK TEST	
			df	F	α	df	F	α	Z	α
Body Mass	M		1,32	0.30	.59	38,1212	0.96	.54		
	F		1,24	0.46	.51	38,906	0.46	.99		
Body Mass Ratio	M		1,32	0.65	.57	38,1212	0.92	.60		
	F		1,24	0.01	.93	38,906	0.15	1.00		
Growth Rate	M		1,32	0.62	.56	37,1180	0.68	.93		
	F		1,24	0.58	.54	37,881	1.43	.05		
Sitting Height	M		1,32	0.03	.87	38,1204	0.71	.91		
	F		1,24	0.85	.63	38,904	0.64	.95		
Shoulder Width	M		1,32	0.00	.96	38,1204	0.65	.95		
	F		1,24	1.09	.31	38,904	0.70	.92		
Hip Width	M		1,32	0.12	.73	38,1202	0.59	.98		
	F		1,24	1.88	.18	38,904	1.04	.41		
Head Length	M		1,32	0.41	.53	38,1204	0.82	.77		
	F		1,24	1.26	.27	38,904	0.55	.99		
Head Width	M		1,32	0.21	.66	38,1204	2.63	<.001		
	F		1,24	0.02	.89	38,904	0.48	.99		
Knee Height	M		1,32	0.46	.51	38,1203	0.74	.87		
	F		1,24	2.79	.11	38,904	1.27	.13		
Upper Leg Length	M		1,32	0.94	.66	38,1204	0.64	.96		
	F		1,24	1.79	.19	38,904	1.41	.05		

PARAMETER	SEX	TRANSFORMATION CODE *	DIFFERENCE BETWEEN MEAN (ANOVA)			GROUP VS TIME INTERACTION (ANOVA)			RANK TEST	
			df	F	α	df	F	α	Z	α
Lower Arm Length	M		1,32	0.01	.90	38,1205	0.48	.99		
	F		1,24	0.81	.62	38,904	0.48	.99		
Leukocytes (WBC)	M	1	1,32	0.36	.56	33,1031	1.05	.13		
	F	1	1,24	0.19	.89	33,753	1.29	.13		
Neutrophils	M		1,32	0.38	.84	33,1000	0.79	.79		
	F		1,24	0.49	.50	33,726	0.66	.93		
Lymphocytes	M		1,32	0.50	.82	33,1000	0.85	.72		
	F		1,24	0.24	.63	33,726	0.53	.99		
Monocytes	M								1.02	.31
	F								0.49	.63
Eosinophils	M								0.19	.85
	F								0.80	.43
Basophils	M								0.36	.72
	F								-0.54	.59
Bands	M								1.08	.28
	F								0.33	.74
Erythrocytes (RBC)	M		1,32	0.76	.61	33,1031	0.86	.70		
	F		1,24	0.01	.93	33,752	0.94	.60		
Hematocrit	M		1,32	1.60	.21	33,1031	0.61	.96		
	F		1,24	0.03	.87	33,752	0.89	.65		
Hemoglobin	M		1,32	1.92	.17	33,1031	0.68	.92		
	F		1,24	0.10	.75	33,752	0.89	.65		

PARAMETER	SEX	TRANSFORMATION CODE*	DIFFERENCE BETWEEN MEAN (ANOVA)			GROUP VS TIME INTERACTION (ANOVA)			RANK TEST	
			df	F	α	df	F	α	Z	α
MCV	M		1,32	0.04	.85	33,1030	0.52	.99		
	F		1,24	0.11	.74	33,750	0.48	.99		
E2, 106-156 Weeks	F		1,24	0.08	.77	50,1178	1.48	.02		
E2, 157-234 Weeks	F		1,24	0.03	.86	64,1493	0.82	.85		
T, 120-156 WKS; AM	M		1,32	0.05	.81	11,347	1.13	.33		
T, 120-156 WKS; PM	M		1,32	0.93	.66	11,344	0.39	.96		
T, 157-234 WKS; AM	M		1,32	0.37	.56	26,778	1.73	.01		
T, 157-234 WKS; PM	M		1,32	0.00	.98	25,763	1.56	.04		
DHT, 120-156 WKS, ALL	M		1,32	1.87	.18	35,1087	0.92	.61		
DHT, 157-234 WKS, AM	M		1,32	0.02	.89	25,674	0.87	.65		
DHT, 157-234 WKS, PM	M		1,32	0.02	.89	14,387	0.57	.89		

*1 = Square Root

statistically significant ($\alpha > 0.05$). There also were no significant differences between the mean estradiol (E2) levels of exposed and control females (Figure 17), in the number of ovulatory menstrual cycles of the two groups of females, or in the total number of menstrual cycles observed in both groups. However, there was a significant group versus time interaction ($\alpha < 0.02$) for E2 levels over weeks 106 to 156. This interaction is a result of differences in the last half of this interval that did not continue into the subsequent period (157 to 234 weeks), where the interaction was not significant ($\alpha > 0.05$). The relationships of observed menstruation with progesterone and E2 spikes, in semi-weekly blood samples from which ovulations were determined are shown for two representative females in Figures 18 and 19. Note that an E2 surge always preceded a spike in P (resulting from corpus luteal activity after ovulation), and menstruation followed the decline in P levels. These two females clearly demonstrate a seasonal pattern in ovulations that was apparent in all the females. Subject 20 (Figure 18) represents one of a small group of older females that began to ovulate during the fall or winter following their second birthday (1981-82). Four animals of each group (exposed and control) showed such a pattern. Seven of these eight animals, including subject 20, then became anovulatory during the following spring and summer. Subject 35 (Figure 19) is representative of the other females which did not ovulate at all until the fall or winter following their third birthday (1982-83). In fact, no animal was observed to ovulate for the first time during the April to September time frame. One control female never ovulated during this study, although she did have menstrual cycles. The peak month for first ovulation was December. Eight females first ovulated in December. This seasonality was not as strong in the second summer associated with ovulatory activity. Ten of the 25 females continued to ovulate regularly during the spring and summer in which they turned 4 years old (1983). Menstruation was not as seasonal in either its onset or its pattern following its first occurrence. The month of menarche showed no seasonal peak or lull among these animals. Only slight reductions in the frequency of menstruation were observed in the spring and summer periods during which ovulatory activity diminished.

For the males, the sharp rise in circulating testosterone levels that is a marker event for the onset of puberty occurred abruptly at a particular age for each male (Table VI). The onset and development of this pattern is shown for one animal in Figure 20. As the animals matured, both morning and evening levels of testosterone showed additional increases (Figures 21 and 22). Note that the age at which testosterone levels began to rise corresponds to the age at which the growth curve of the exposed group began to cross over the growth curve of the control group (Figures 6 and 21). During the early period of increasing testosterone levels, the exposed group showed a trend toward higher mean testosterone levels than those in control males (Figures 21 and 22). The differences in mean values were not statistically significant (Table V), but the interaction term for both AM and PM levels was significant for the period of 157 to 234 weeks of age. This significant interaction term indicates that the testosterone levels in the two groups were not changing with time in an equivalent manner. However, since no consistent trend is apparent for this period (157 to 234 weeks), the interaction term may be a result of random variation. Thus, the differences in testosterone levels probably have

little or no physiological significance. No significant differences in dihydrotestosterone (DHT) levels between the groups were observed.

TABLE VI

Occurrence of Key Events in Sexual Development[a]

	Females First Menstruation	Females First Ovulation[b]	Males First Testosterone Spike[b]
Control	122.1 ± 5.2	169.4 ± 6.2	142.0 ± 3.4
Exposed	119.2 ± 4.3	168.2 ± 6.2	136.9 ± 3.4

[a]All values are expressed as mean ± SE in weeks of age.

[b]Determined from hormone measurements. Testosterone spikes were noted in night (2200) samples.

No statistically significant differences were found between exposed and control animals of either sex for the 11 hematological parameters measured (Table V). Data for seven of these parameters are shown in Figures 23 to 29. Observations of monocytes, eosinophils, basophils, or bands were uncommon and occurred equally in the exposed and control animals and for males and females. The average number of monocytes or eosinophils noted was in the range of 0 to 2 percent and the average number of basophils or bands noted was always less than 0.5 percent.

In general, the health of all of the monkeys in this project, exposed or control, was excellent. Minor problems common in rhesus monkey colonies were noted in both groups, including abrasions, regional alopecia, gingivitis, and hematoma. No substantive differences between the two groups were noted. Intradermal skin tests for tuberculosis were done every 6 months; all results were negative. Bacterial enteritis was also periodically evaluated with rectal swab and culture. No enteric pathogens were identified in these animals.

Tooth eruption was noted during each physical examination. An interim report on this data has been published (6). No differences between groups were found in the mean age at which permanent teeth erupted.

Soon after they reached 54 months of age, males were transferred to the NAMRL vivarium where they were housed in standard wire primate cages until they were transferred to other Department of Defense laboratories for use in other projects. One male from the control group died during this period due to a progressive emaciation of unknown etiology. That animal had been carefully observed and repeatedly had fecal samples cultured during the last 2 years of the project because of recurrent diarrhea, but

no etiology for his intestinal problems was ever discovered. The females remained in the ELF facility until the end of the exposure period in June 1984, when the youngest male reached 54 months of age. The females were subsequently transferred to the NAMRL colony where they were housed in small groups until they were later transferred to another laboratory for use. Two females from the exposed group died during this transition period from complications secondary to injuries from fighting.

DISCUSSION

As a followup study, the purposes of this project were to replicate ELF1, validate the growth rate enhancement finding, and if it reoccurred, to determine the process that caused this growth rate change. From a statistical viewpoint, ELF2 did not replicate the finding of ELF1 that chronic ELF exposure enhanced the growth rate in pubescent male rhesus monkeys. This study did show, however, that exposed monkeys showed a slightly higher growth rate during puberty than their control group counterparts. Thus, in comparing the two NAMRL studies, both showed a slightly higher growth rate during puberty in ELF exposed males, but the degree of this enhancement was not as large in ELF2. The effect shown in ELF1 remains qualitatively credible in light of ELF2, but was not replicated by ELF2.

As in any other case where an experiment is replicated but the results are different, it is impossible to unequivocally state which finding is valid. Neither experiment invalidates the other, although an effect that cannot be readily reproduced may be considered so weak as to be inconsequential. It may be that the exposure in question is near a threshold intensity for producing the effect, and that higher intensity exposures would reproducibly stimulate growth in males. Since the monkeys were exposed to ELF fields much stronger than those that will be created by the Navy's ELF submarine communications system, no effect on growth would be expected at the ELF intensities associated with the actual antenna.

The influence of other environmental factors, particularly social isolation, on the monkeys in this study is difficult to assess. As noted in the Methods section, great care was taken to see that the exposed and control groups experienced like conditions in every respect. Comparable data from the NAMRL colony from which these monkeys were taken does not exist. Very little data exists in the literature that can provide meaningful comparisons to the NAMRL data. For growth, the data of Van Wagenen and Catchpole (17) covered similar ages for captive born rhesus monkeys at Yale University, but the housing conditions of the animals were not specified. The mean body mass of NAMRL ELF2 monkeys was nearly identical to that reported by Van Wagenen and Catchpole during the first 2 years of life. Our animals were somewhat smaller than those at Yale beyond 2 years of age. At 3 and 4 years of age, the mean for our animals (male or female) was about the value of one standard deviation below the mean for the Yale monkeys. The hematology results and veterinary examinations for the monkeys in this study indicate that the animals were in excellent health and not under any unusual stress. The differential WBC counts, which are classic indicators of stress, are consistent with a lack of stress among the monkeys.

Of the three hypotheses set forth as plausible explanations of the ELF1 growth rate effect, only two are possibly compatible with the results of ELF2. Since no differences in growth rate were seen in prepubertal monkeys of either sex, the hypothesis of a generalized anabolic effect can be rejected. Even the small differences in male growth rate observed in ELF2 seemed to be dependent on the higher testosterone levels of puberty. It is still difficult to evaluate the two hypotheses regarding neuroendocrine function. The trend toward slightly higher growth rates in exposed pubescent ELF2 males was coincident with the onset of increased circulating testosterone levels in puberty. In the absence of measurable differences in the testosterone levels of the two groups, there is nothing to directly correlate hormone levels to growth rate in this study. We do not believe that analysis of serum samples for luteinizing hormone is likely to clarify the picture, either. Considerable evaluation of endocrine rhythms in both males and females failed to indicate any aspect of reproductive endocrinology that was effected by ELF exposure. Not only were overall numbers of menstrual cycles in females equal in exposed and control monkeys, but seasonal patterns of ovulation in the female rhesus were similar between groups. The presumed seasonal aspect of ovulatory patterns was especially interesting in view of the fact that these animals were in rigidly controlled light and temperature conditions from birth. We do not think that the significant interactions in E2 and T levels noted in Table V have any profound meaning for the study. These are more likely to be an artifact resulting from the rhythmic, and thus highly variable, nature of the hormone levels, and from the fact that our system could not accommodate the raw data for the entire period in question. Indeed, the interactions noted did not carry over into (or from) the adjacent period analyzed. The possible physiological significance of an E2 interaction for females is strongly overshadowed by the similarities in menstrual and ovulatory activity of the two groups. For the males, the interactions were created by oscillating levels of testosterone that, over the entire period, showed control values higher than exposed values at times and lower at other times. This variability tends to discount any systematic effect that would have physiological, in addition to statistical, significance.

Because of the observed habit of the male monkeys to sit on the conducting bars of their cages with their scrotums in contact with those bars, we gave considerable attention to the possibility of direct stimulation of the testes by electrical current in ELF1. The monkeys in ELF2 also spent a considerable amount of time in a similar posture, sitting on the bars of the cage. We do not know if there were any quantitative differences in the time spent with the scrotum in contact with the bars during the two studies. Thus, the statistically significant growth effect found in ELF1 males, and the qualitatively similar trends observed in ELF2 males, are consistent with possible direct current stimulation of testicular secretion of testosterone.

Based on these two long-term studies of rhesus monkeys at NAMRL, no effects of ELF exposure associated with the submarine communications systems have been observed that appear to be detrimental to the health of the animals. The observed differences in growth rate of males are, at most, very subtle; occurred at field intensities much higher than those existing from the actual antenna; and are not accompanied by any

disturbances of neuroendocrine function for endpoints that we measured. The growth rates of the monkeys in ELF2 are quantitatively similar to growth rates reported for young rhesus monkeys by other investigators (10, 17).

In summary, statistically significant differences in the growth, endocrine, or hematological parameters studied were, with minor exceptions, not found in rhesus monkeys exposed to ELF electric and magnetic fields for the first 54 months of life. Growth rates of body mass were slightly, but not significantly, higher in exposed males during puberty than in control males. A transient trend toward higher circulating testosterone levels in exposed males was also observed at the age corresponding to the divergence of the body mass curves. These findings, while not replicating the prior observation of enhanced growth rate in pubescent male monkeys exposed to ELF fields, did agree qualitatively with the earlier study. However, the results of the second project failed to provide conclusive support for the hypotheses that stimulation of testosterone secretion was the cause of growth enhancement.

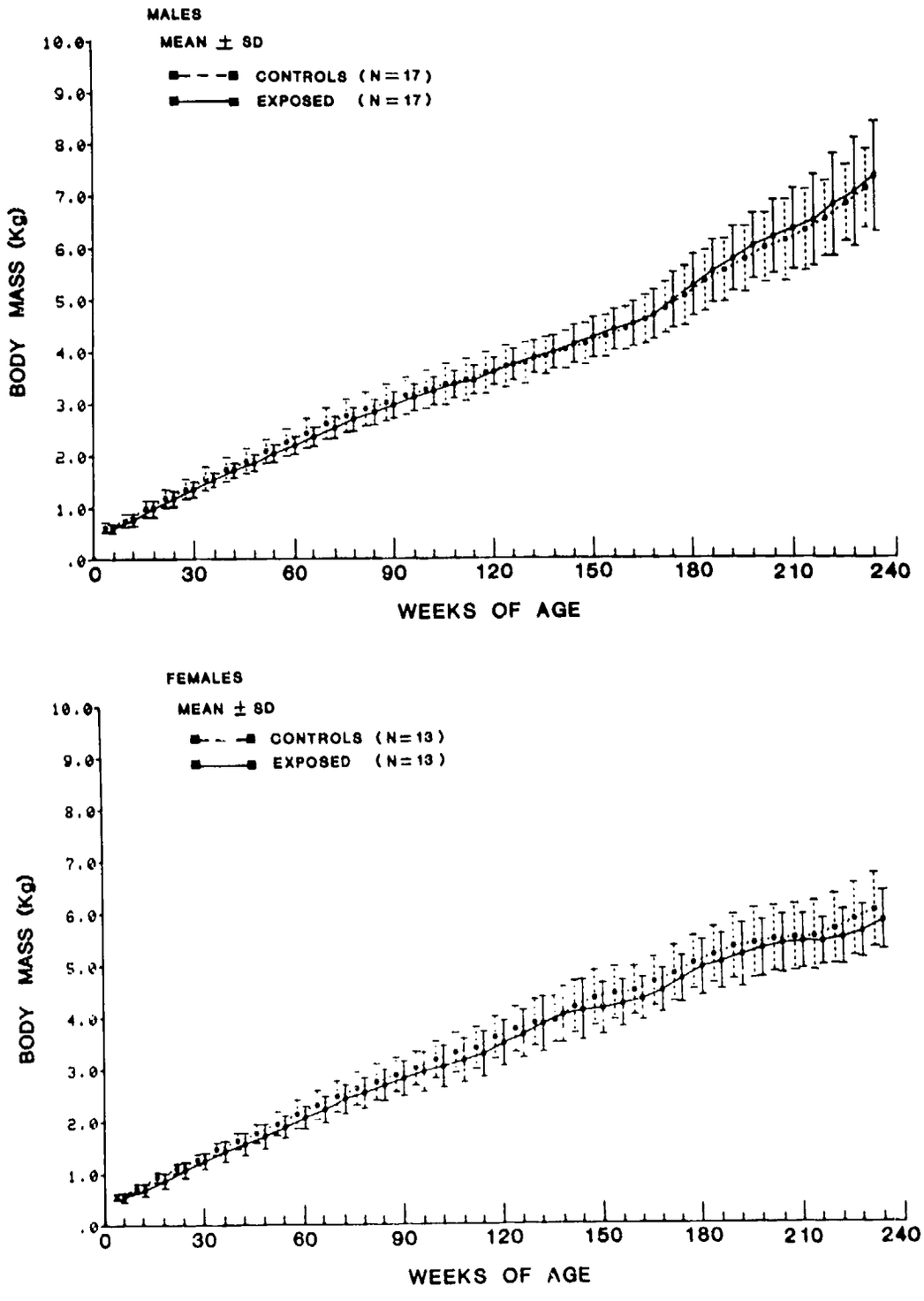
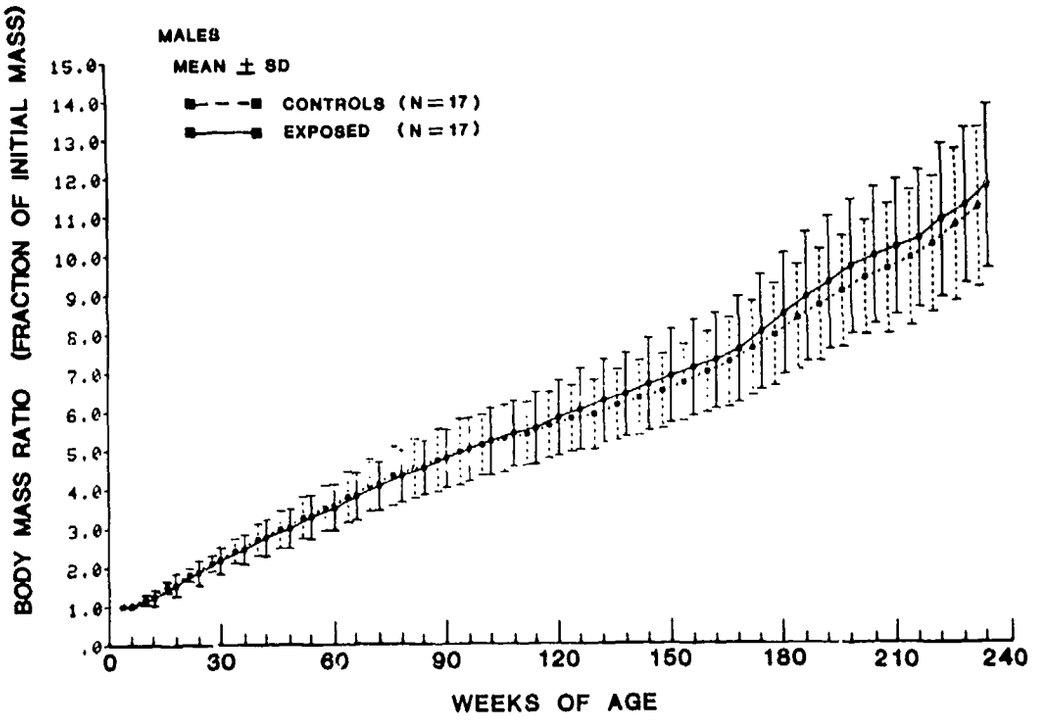


Figure 6

Body mass of control and ELF exposed rhesus monkeys during the first 234 weeks of life. The upper graph in this figure and in Figures 7 to 16 is for males, and the lower graph is for females.



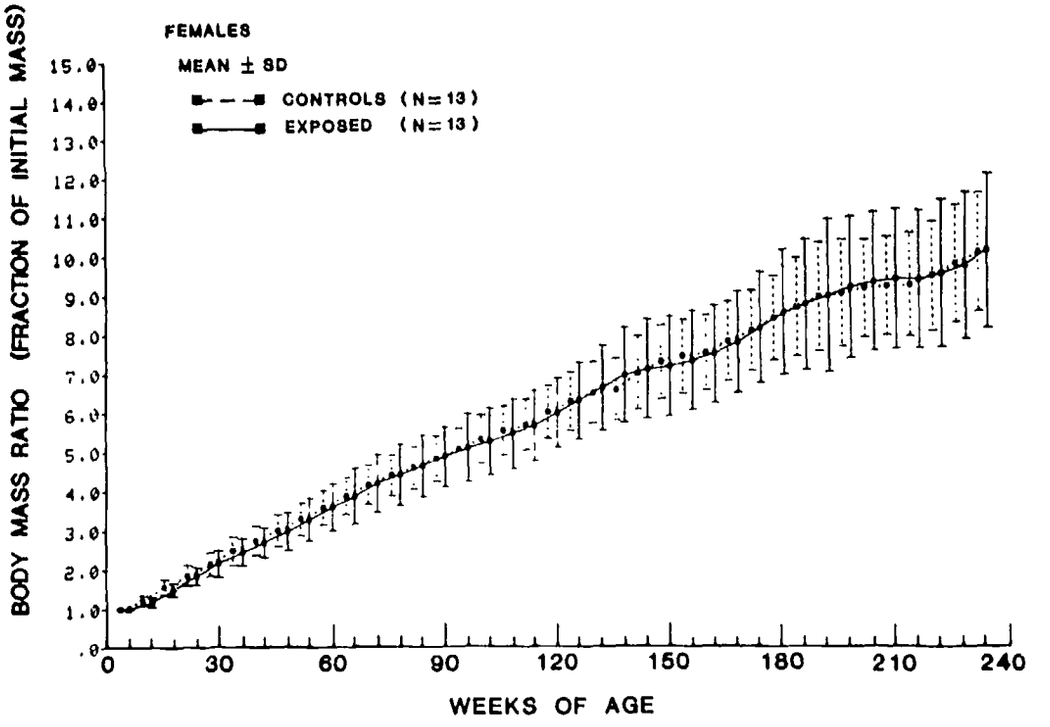
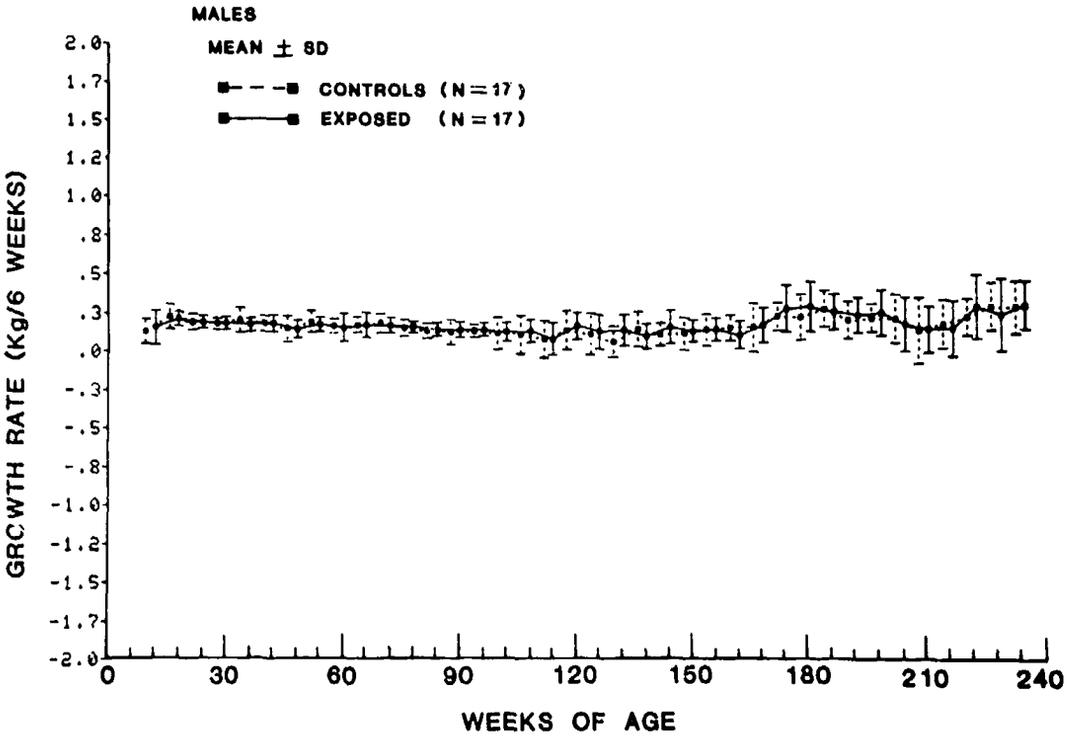


Figure 7

Body mass ratio of control and ELF exposed rhesus monkeys during the first 234 weeks of life.



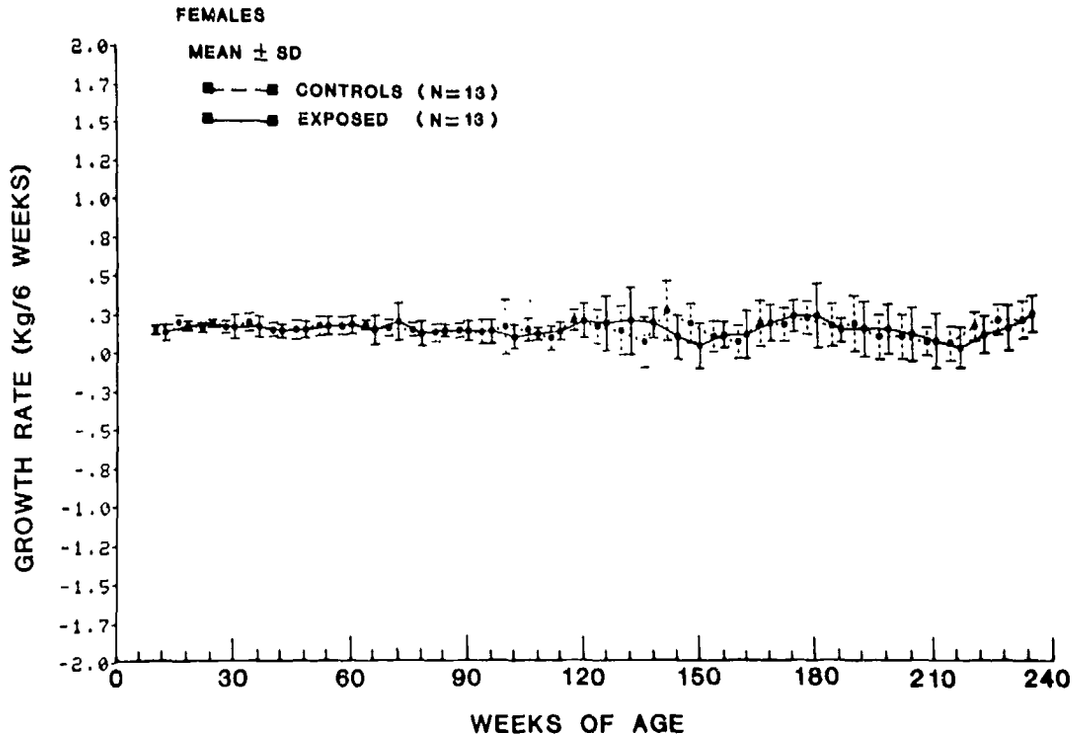


Figure 8

Growth rate of control and ELF exposed rhesus monkeys during the first 234 weeks of life.

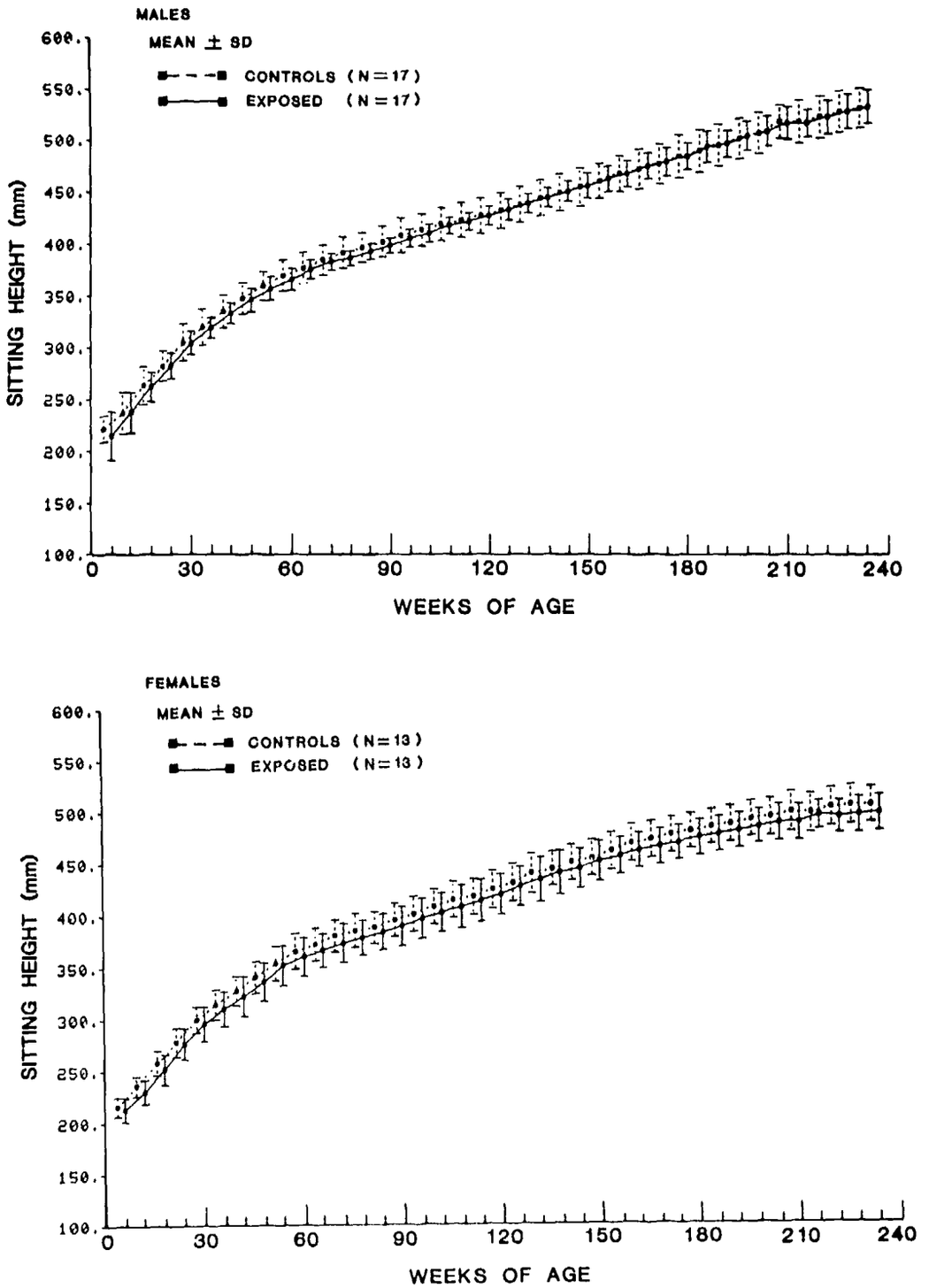


Figure 9

Sitting height of control and ELF exposed rhesus monkeys during the first 234 weeks of life.

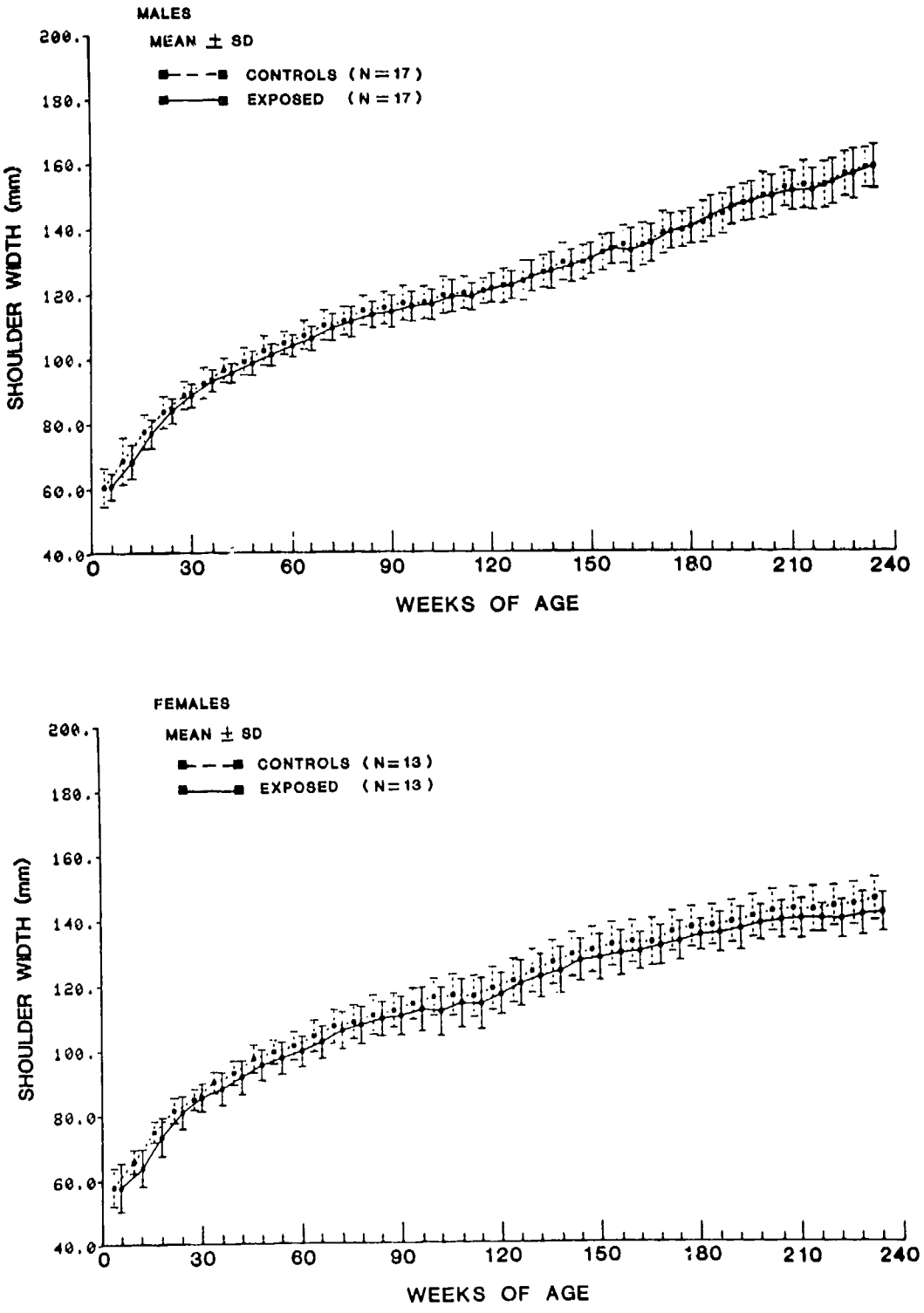


Figure 10

Shoulder width of control and ELF exposed rhesus monkeys during the first 234 weeks of life.

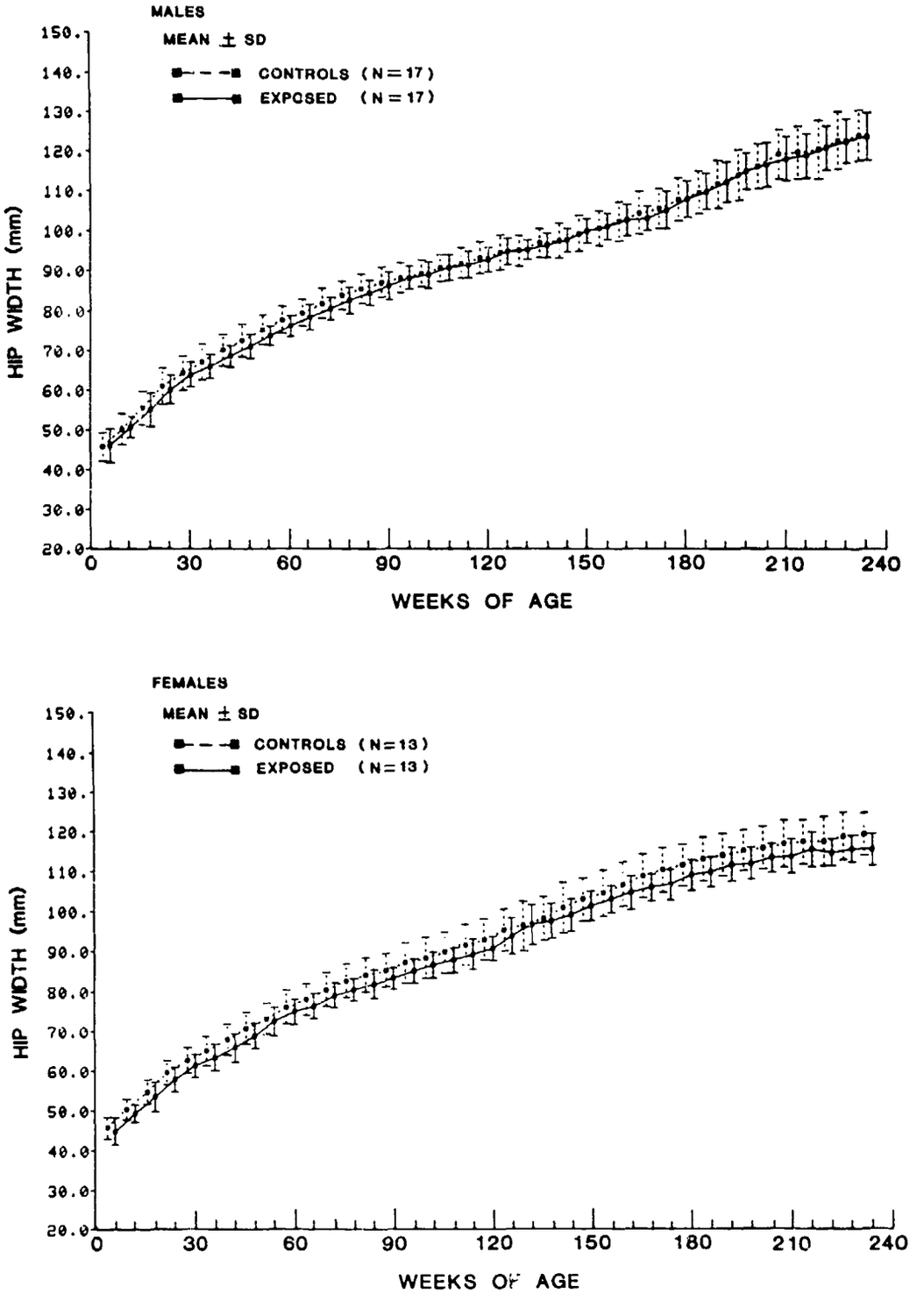


Figure 11

Hip width of control and ELF exposed rhesus monkeys during the first 234 weeks of life.

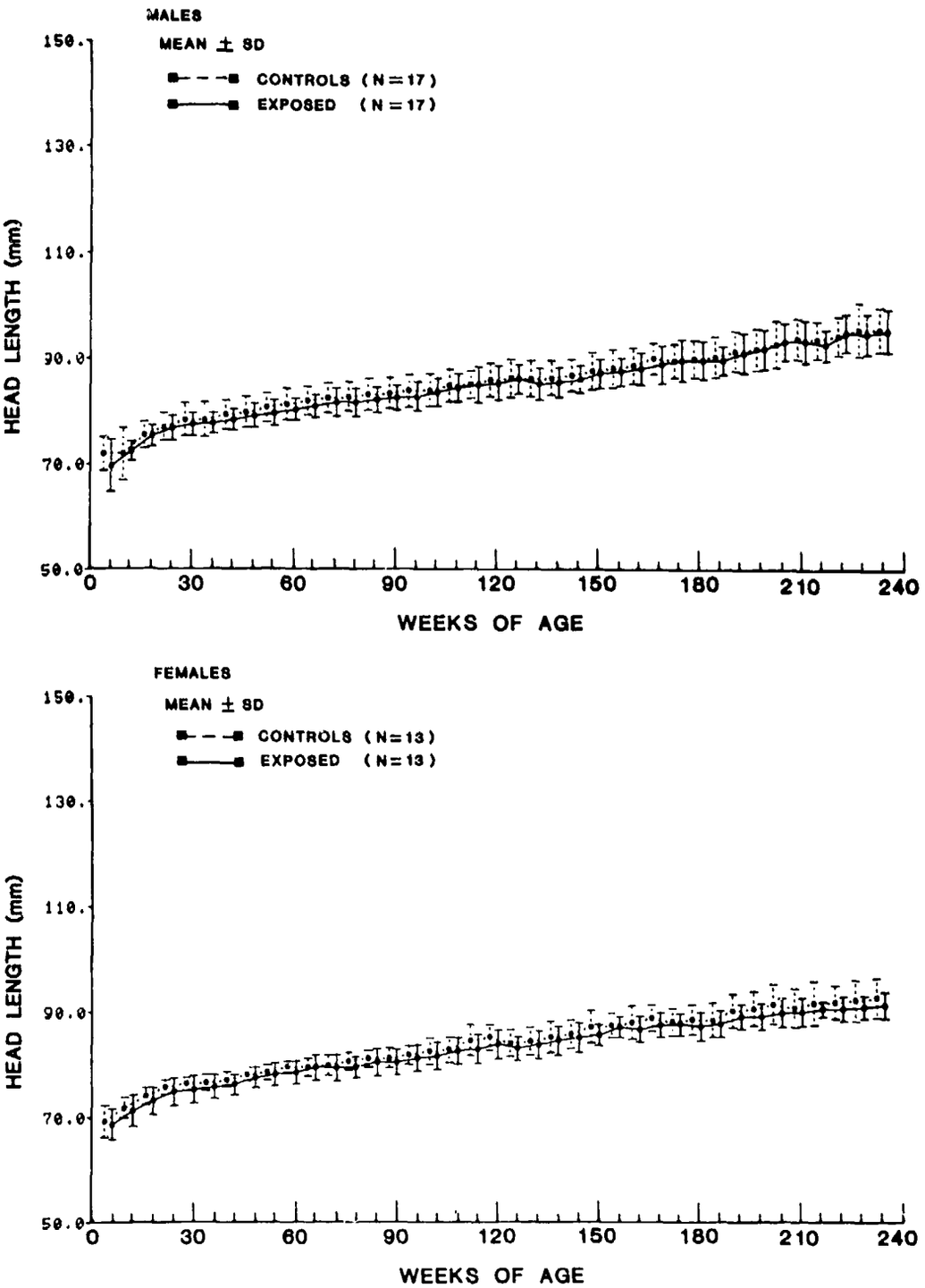
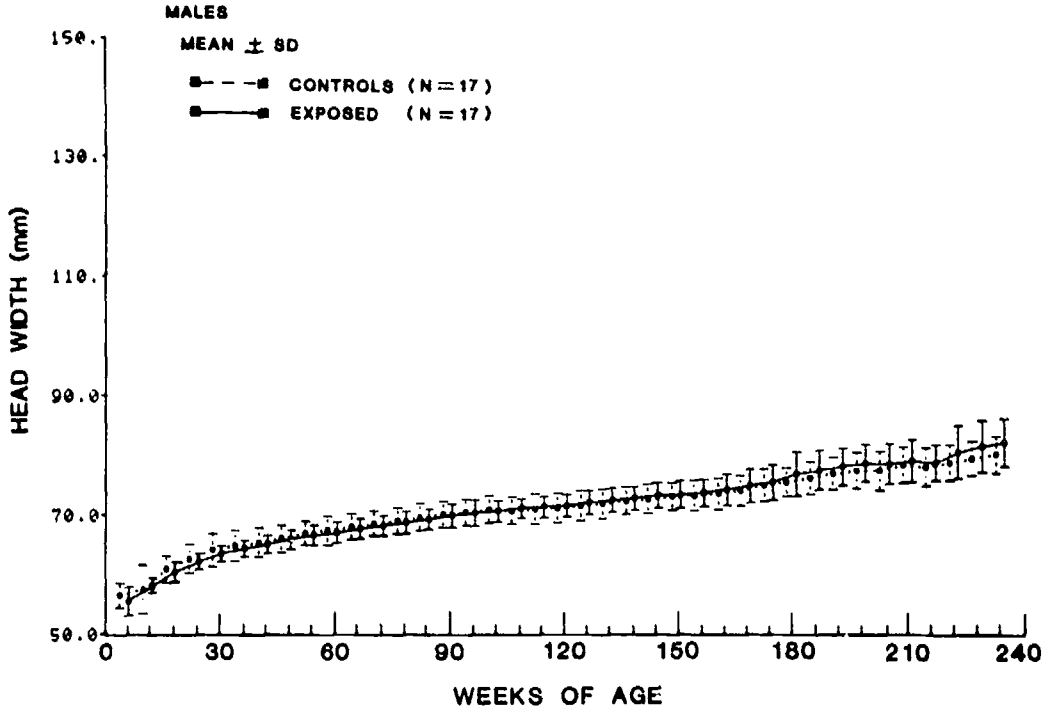


Figure 12

Head length of control and ELF exposed rhesus monkeys during the first 234 weeks of life.



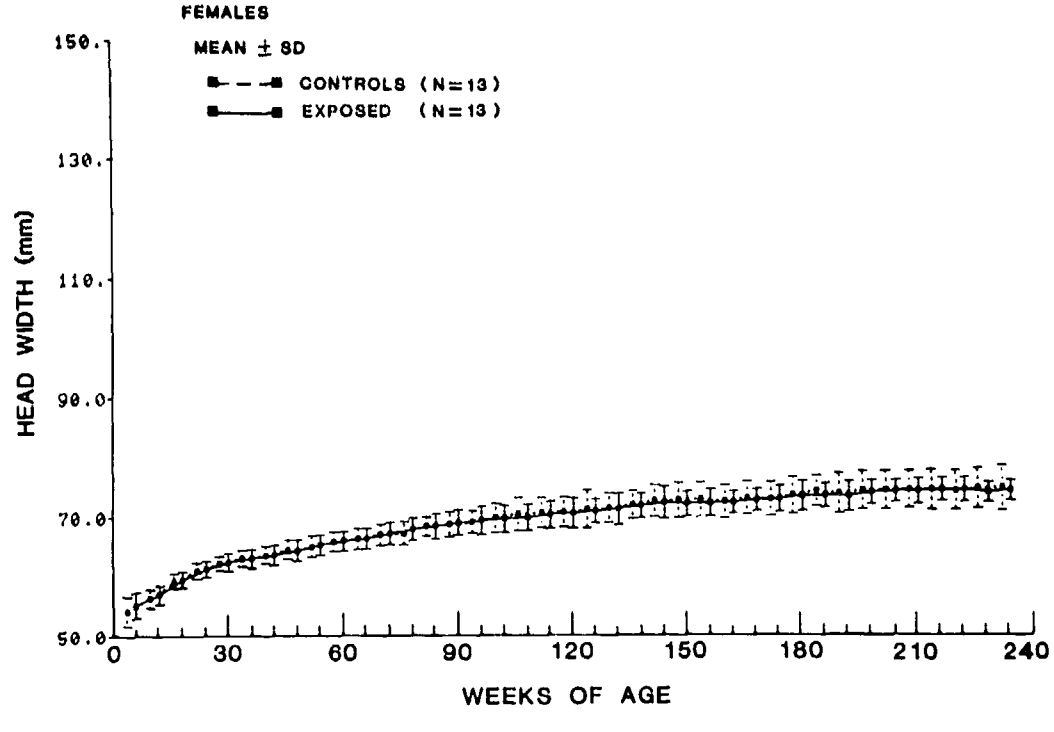


Figure 13

Head width of control and ELF exposed rhesus monkeys during the first 234 weeks of life.

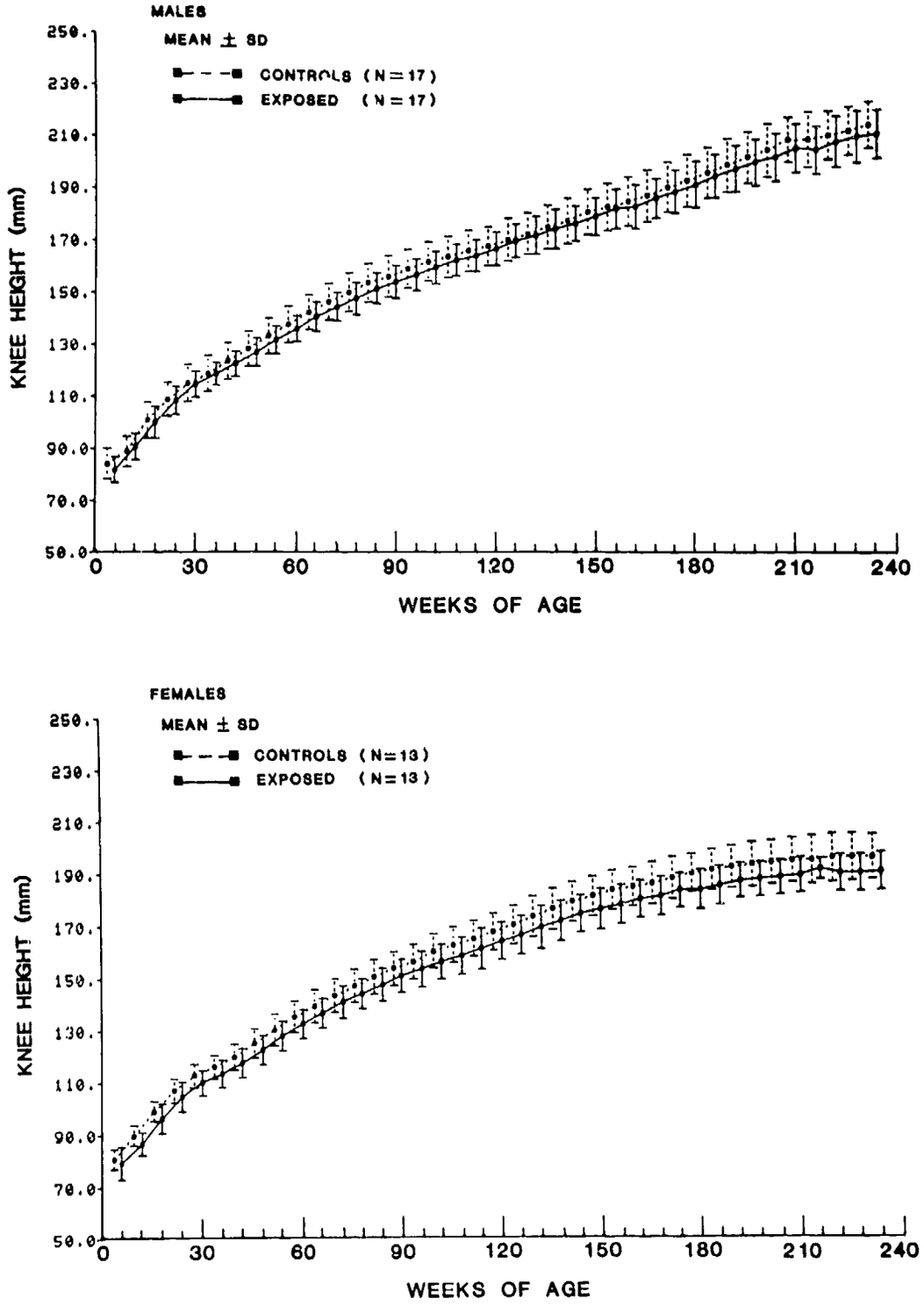
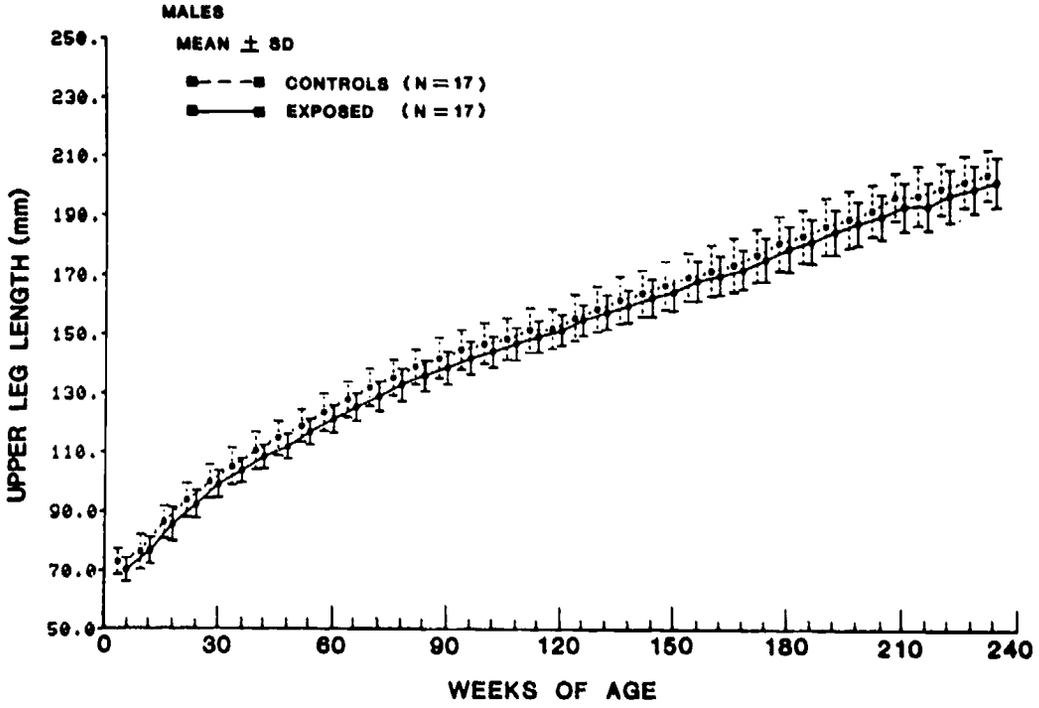


Figure 14

Knee height of control and ELF exposed rhesus monkeys during the first 234 weeks of life.



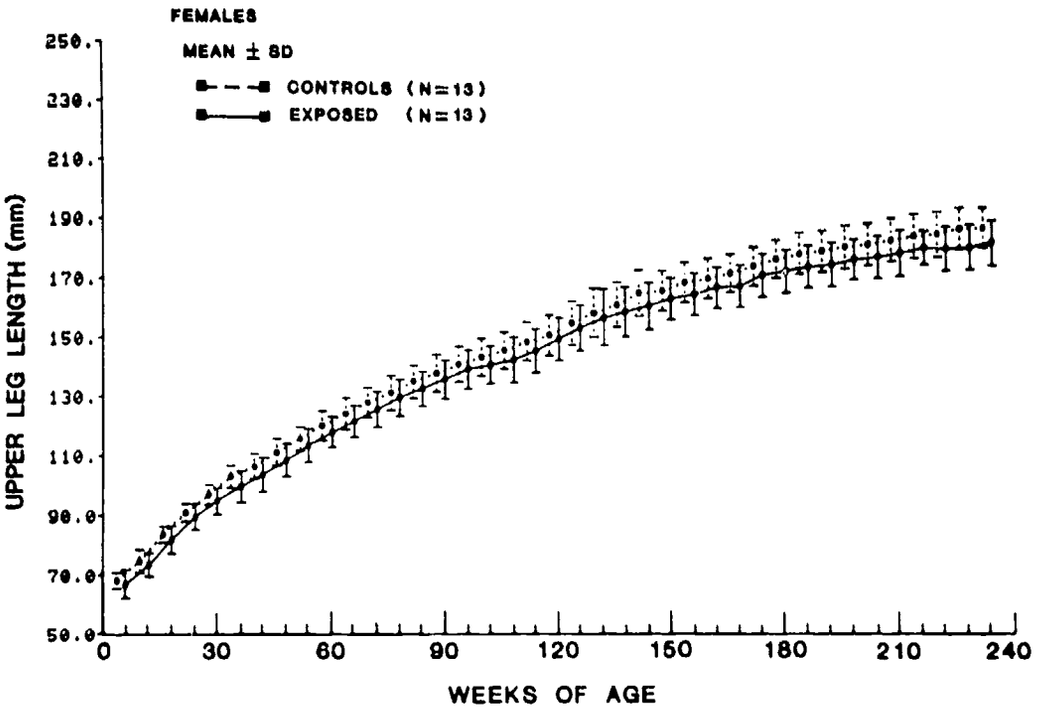
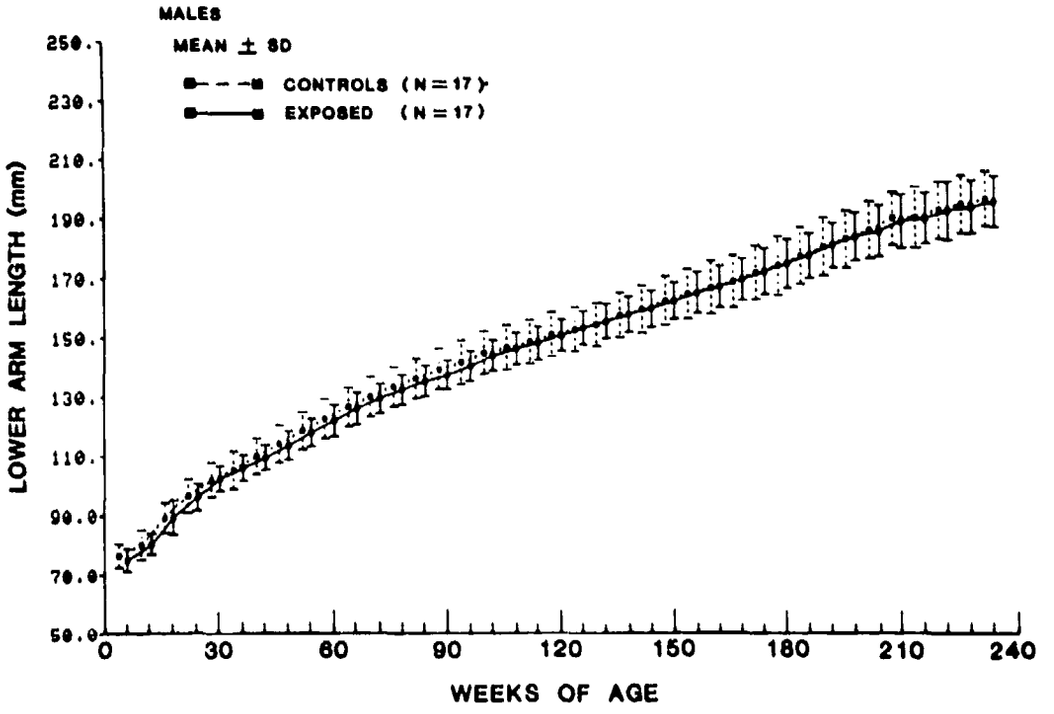


Figure 15

Upper leg length of control and ELF exposed rhesus monkeys during the first 234 weeks of life.



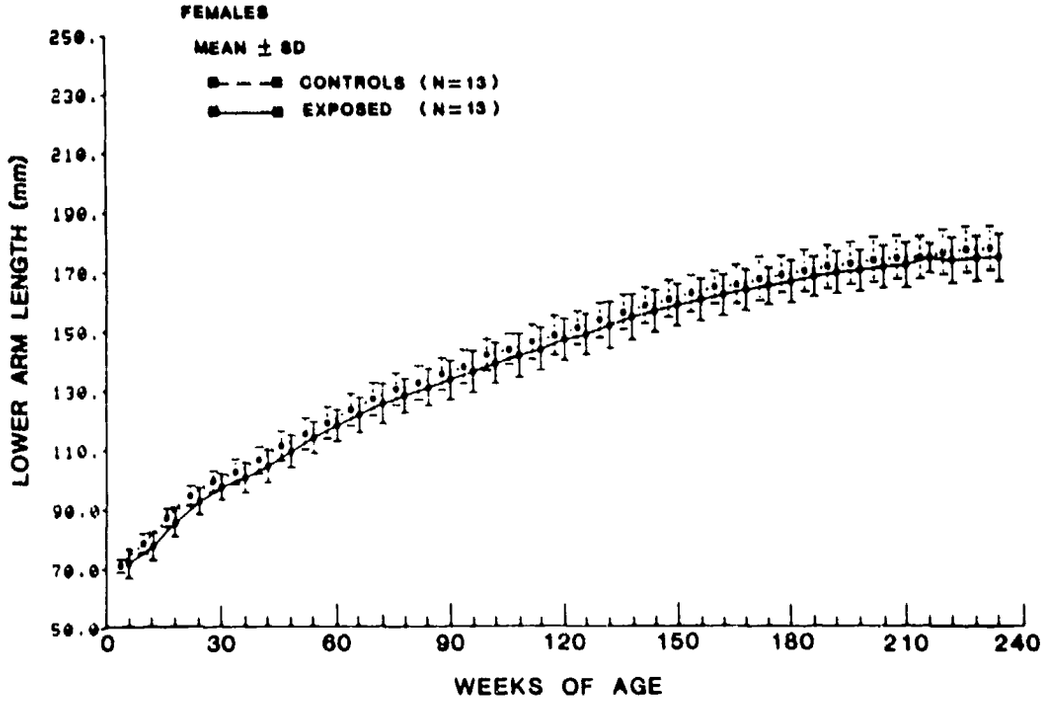


Figure 16

Lower arm length of control and ELF exposed rhesus monkeys furing the first 234 weeks of life.

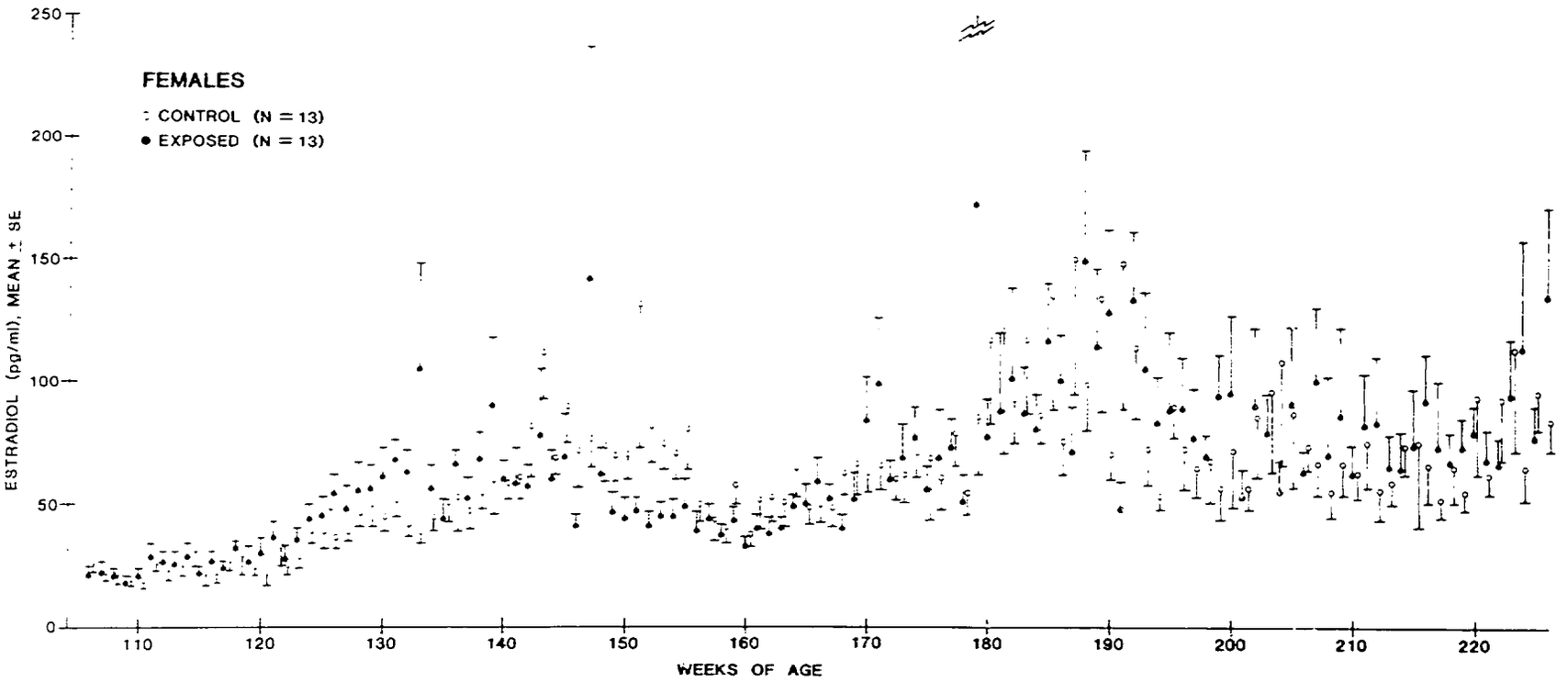


Figure 17

Mean estradiol values (one/week) of control and ELF exposed female rhesus monkeys from 106 to 234 weeks of age. The SE = 102 pg/ml for the point with the discontinuous error bar.

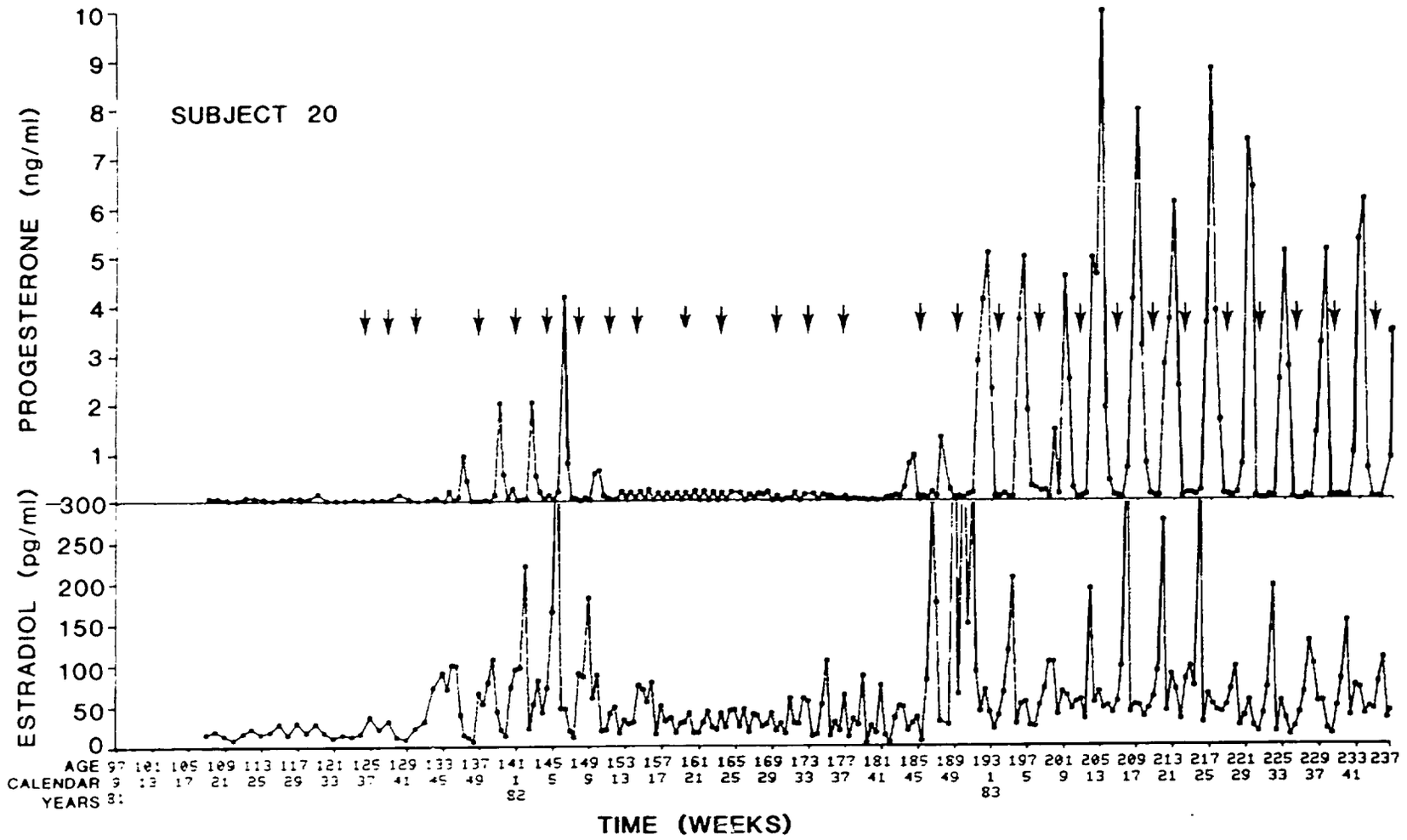


Figure 18

Estradiol and progesterone levels of Subject 20, an ELF exposed female, from 106 to 234 weeks of age. Observed menstruations are indicated by arrows above the progesterone curve. The abscissa has multiple labels to indicate age and calendar weeks.

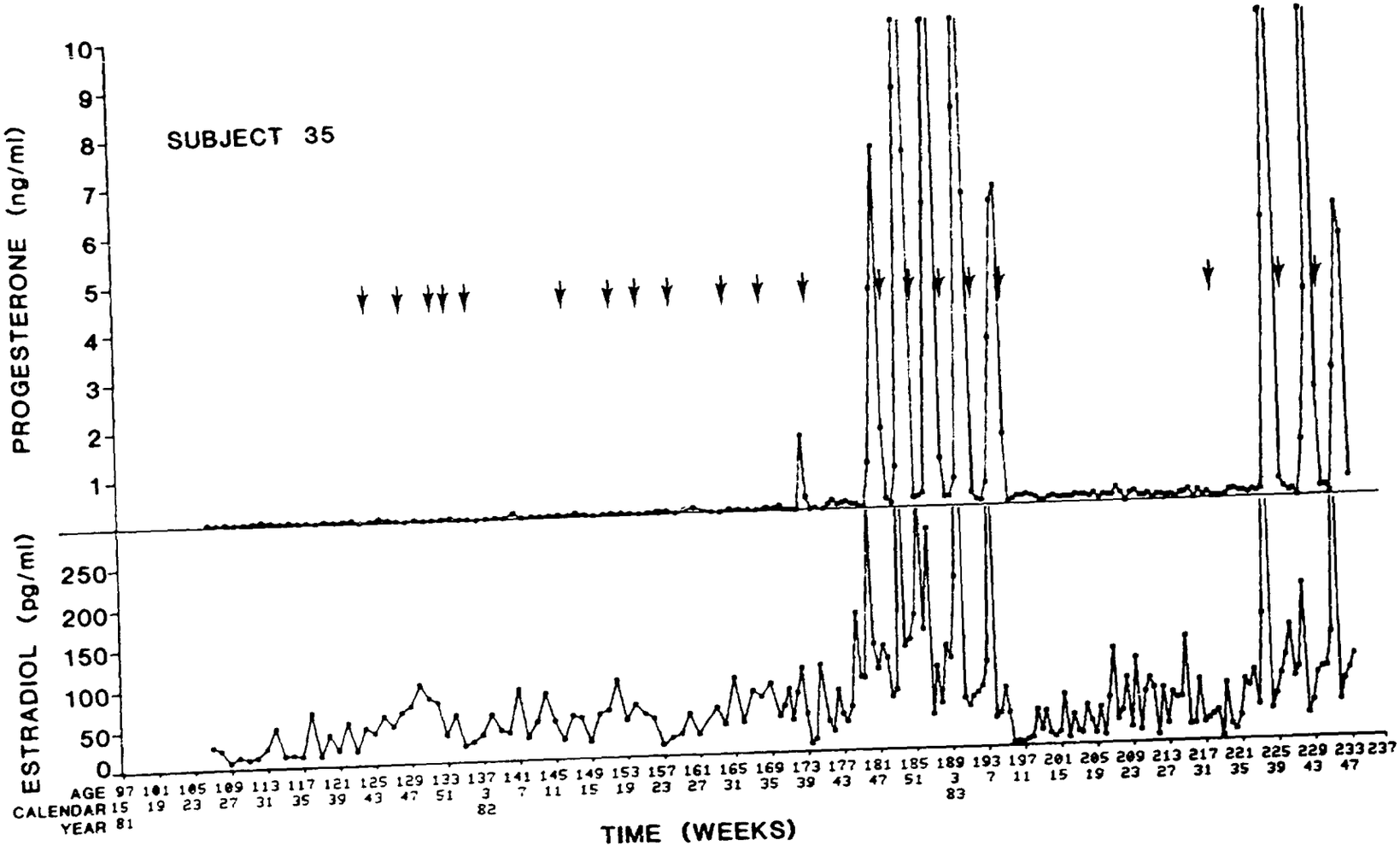


Figure 19

Estradiol and progesterone levels of Subject 35, a control female, from 106 to 234 weeks of age. Observed menstruations are indicated by arrows above the progesterone curve. Abscissa labels are the same as for Figure 18.

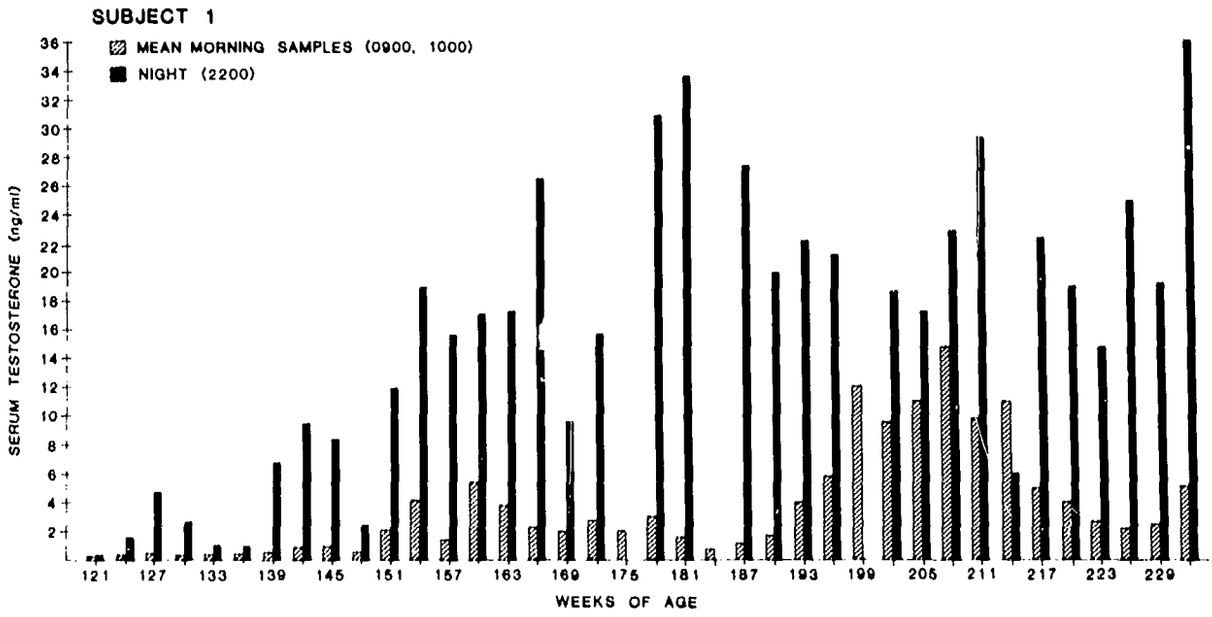


Figure 20

Night and mean morning testosterone levels in Subject 1, an ELF exposed male, from 121 to 234 weeks of age.

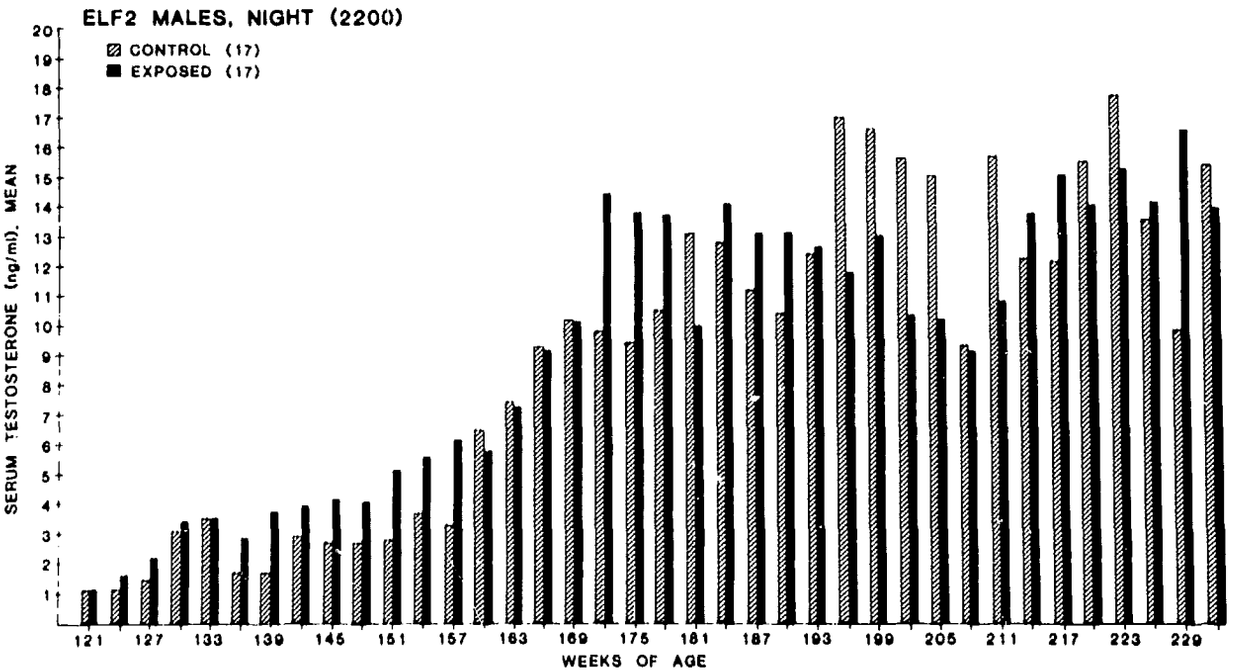


Figure 21

Mean night testosterone levels in control and ELF exposed male rhesus monkeys from 121 to 234 weeks of age. Error bars were omitted for clarity. The standard error of the mean was usually 15 to 20% of the mean value.

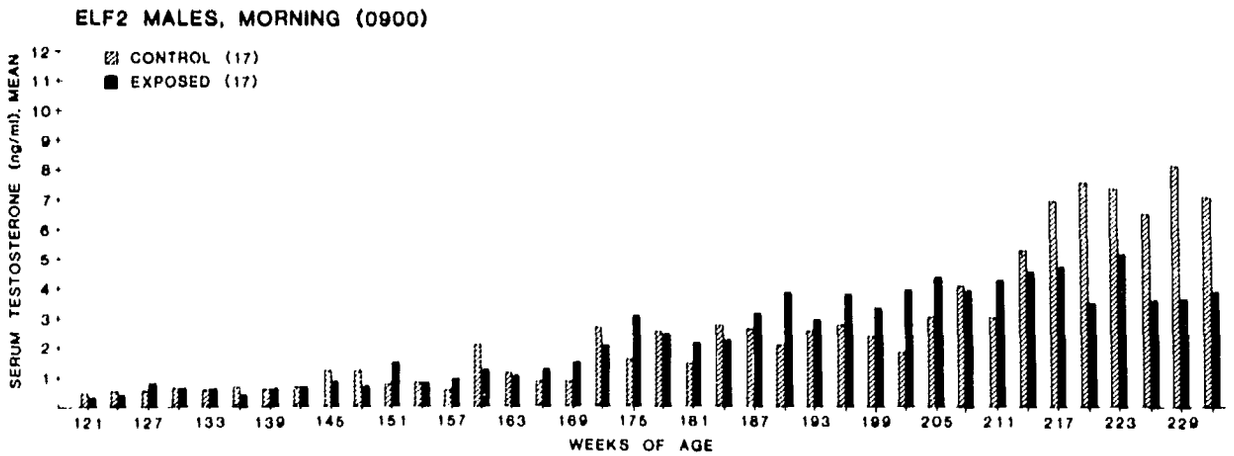


Figure 22

Mean morning testosterone levels in control and ELF exposed male rhesus monkeys from 121 to 234 weeks of age. Error bars were omitted as for Figure 21.

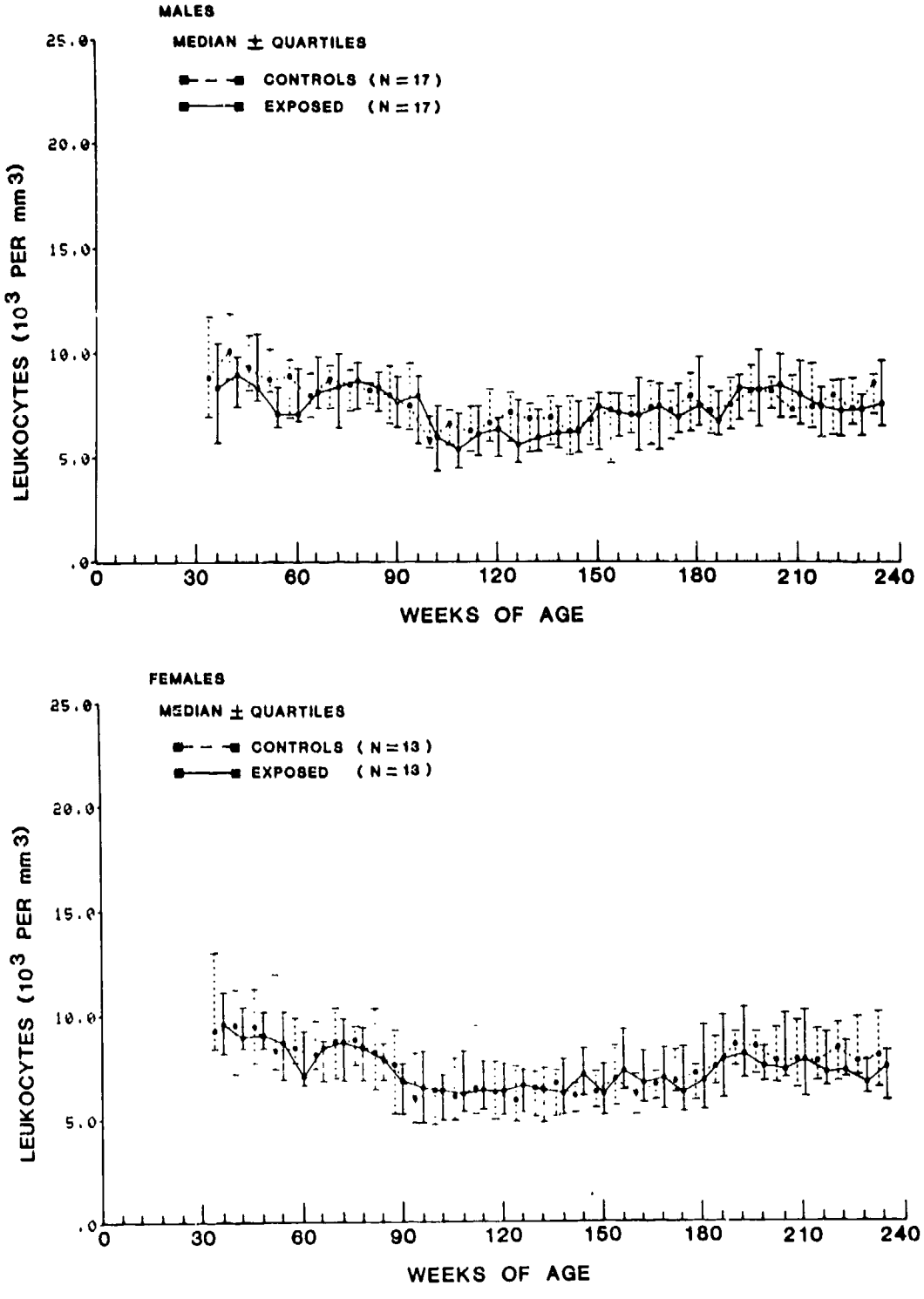


Figure 23

Total leukocyte (WBC) count in control and ELF exposed rhesus monkeys during the first 234 weeks of life. As in Figures 6 to 16, the upper graph in this and all following figures is for males, and the lower graph is for females.

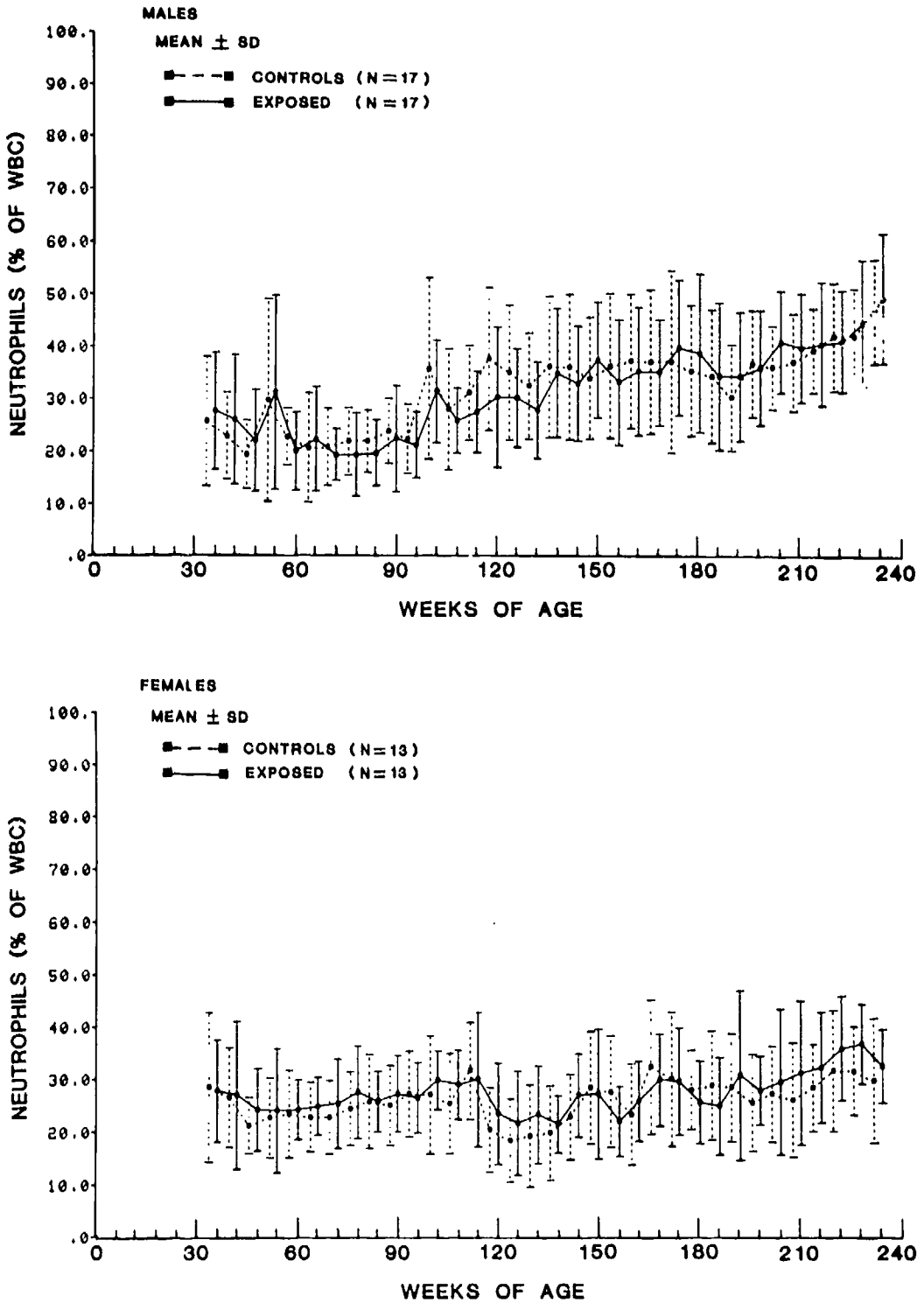


Figure 24

Neutrophil percentage of WBC count in control and ELF exposed rhesus monkeys during the first 234 weeks of life.

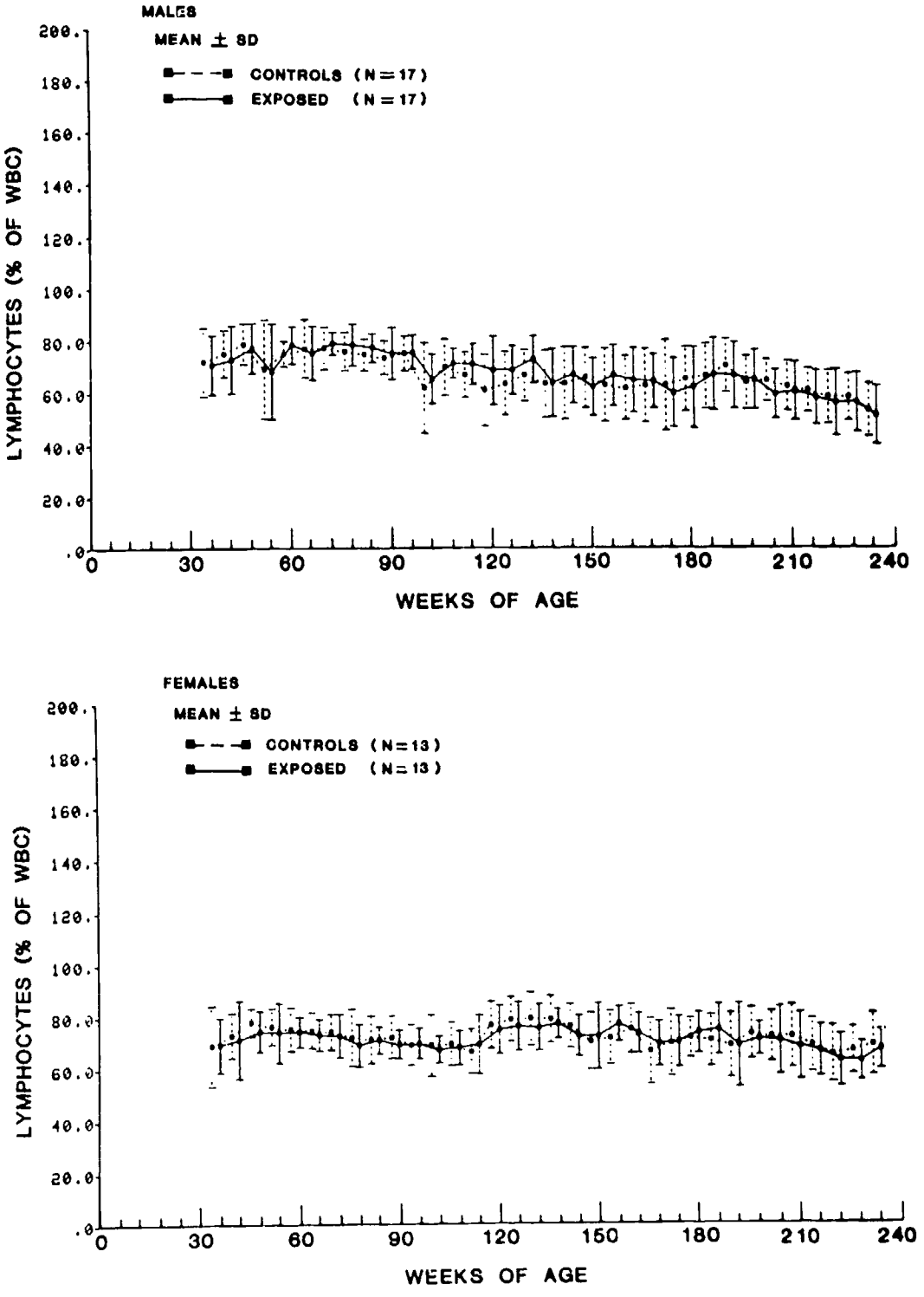


Figure 25

Lymphocyte percentage of WBC count in control and ELF exposed rhesus monkeys during the first 234 weeks of life.

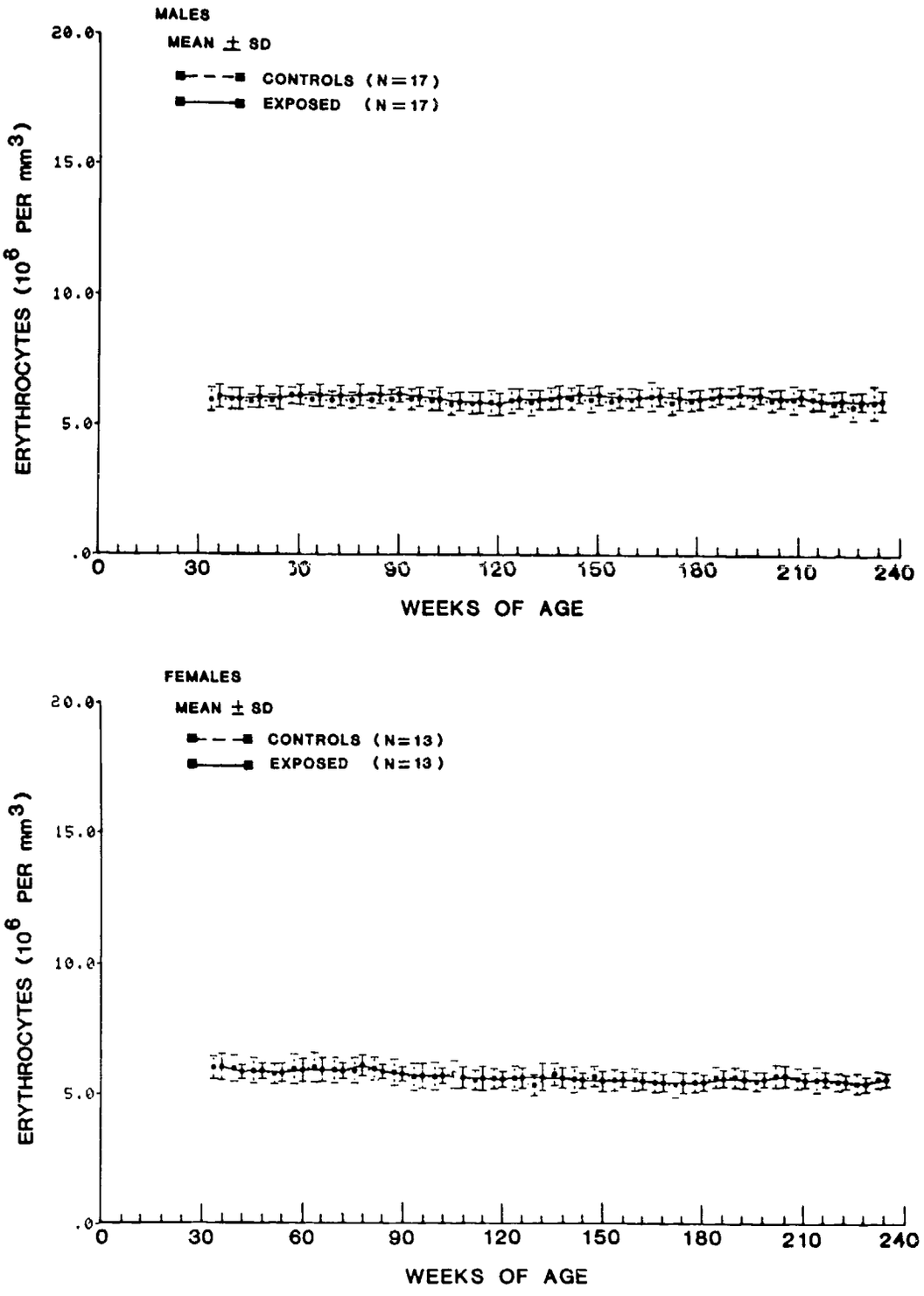


Figure 26

Erythrocyte (RBC) count in control and ELF exposed rhesus monkeys during the first 234 weeks of life.

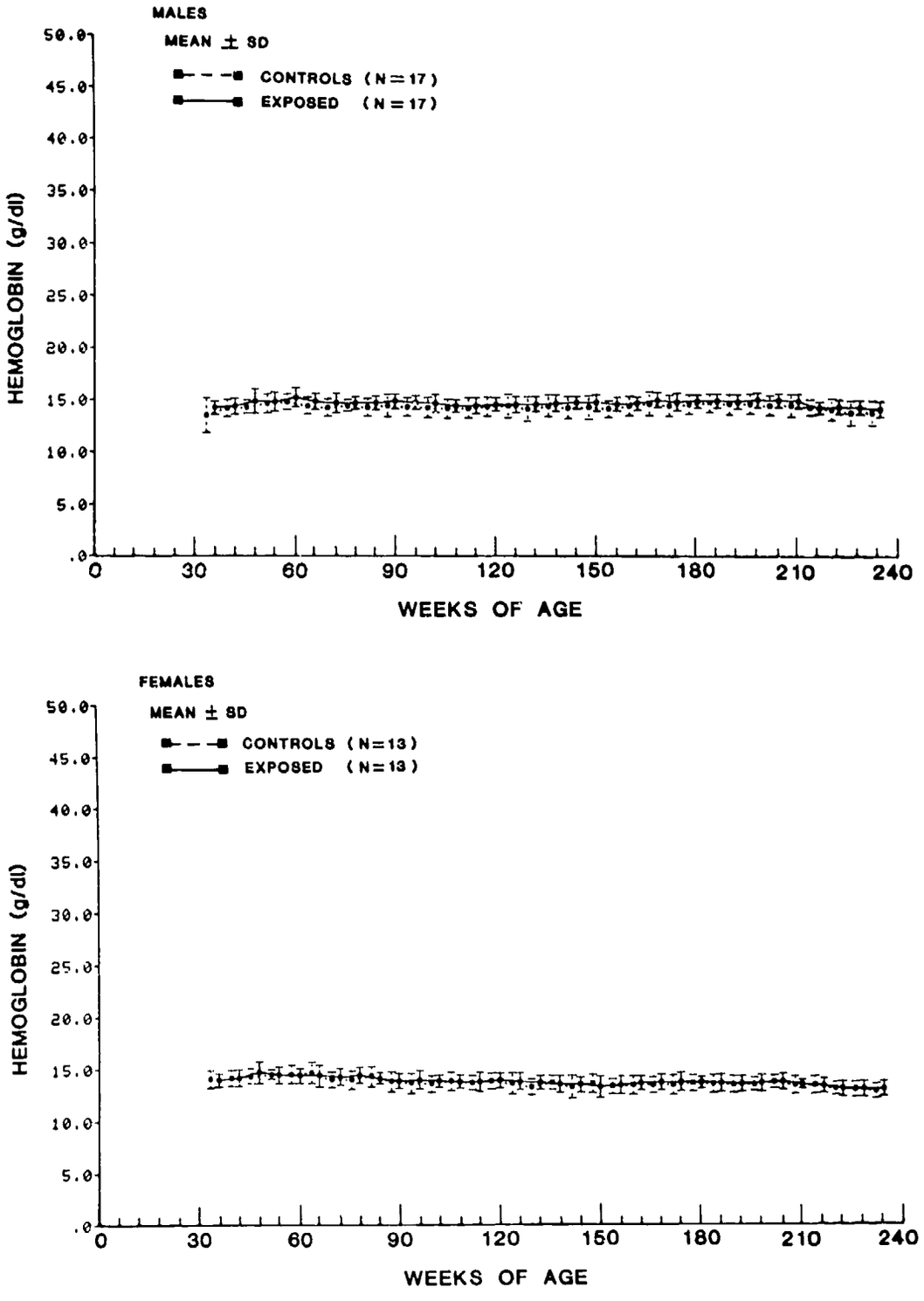
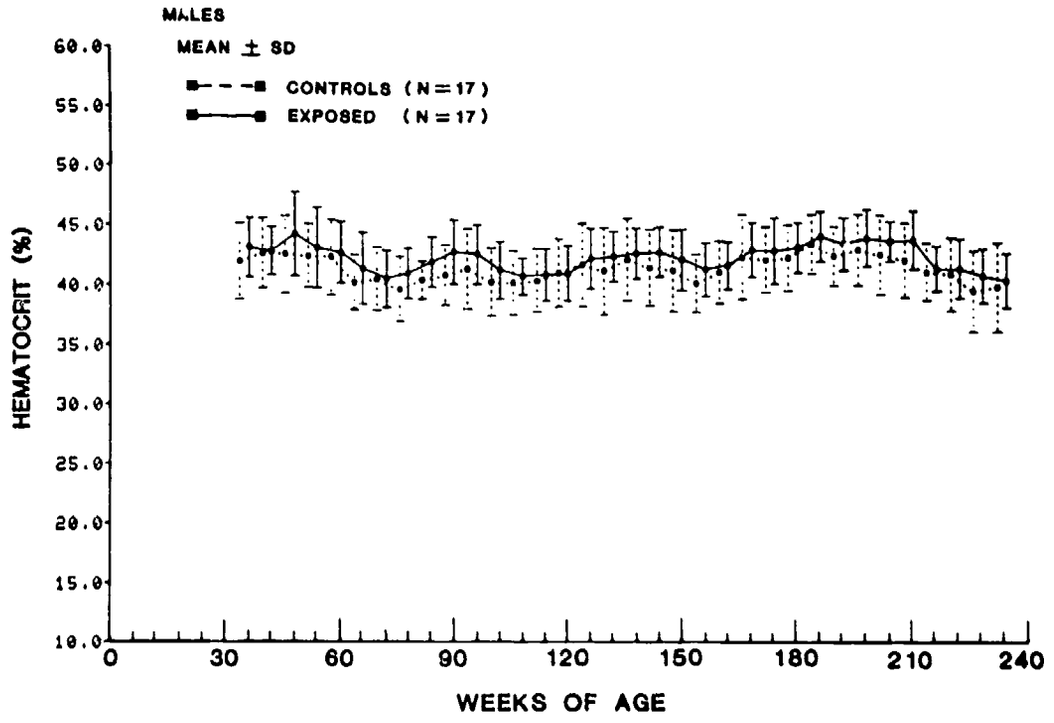


Figure 27

Hemoglobin concentration in control and ELF exposed rhesus monkeys during the first 234 weeks of life.



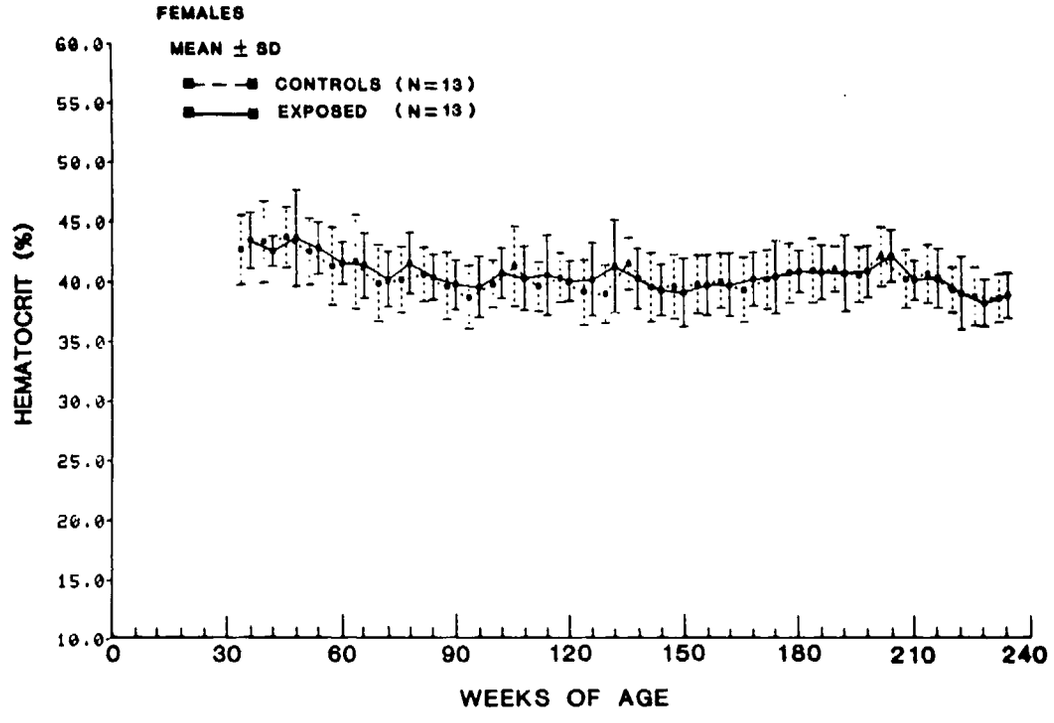
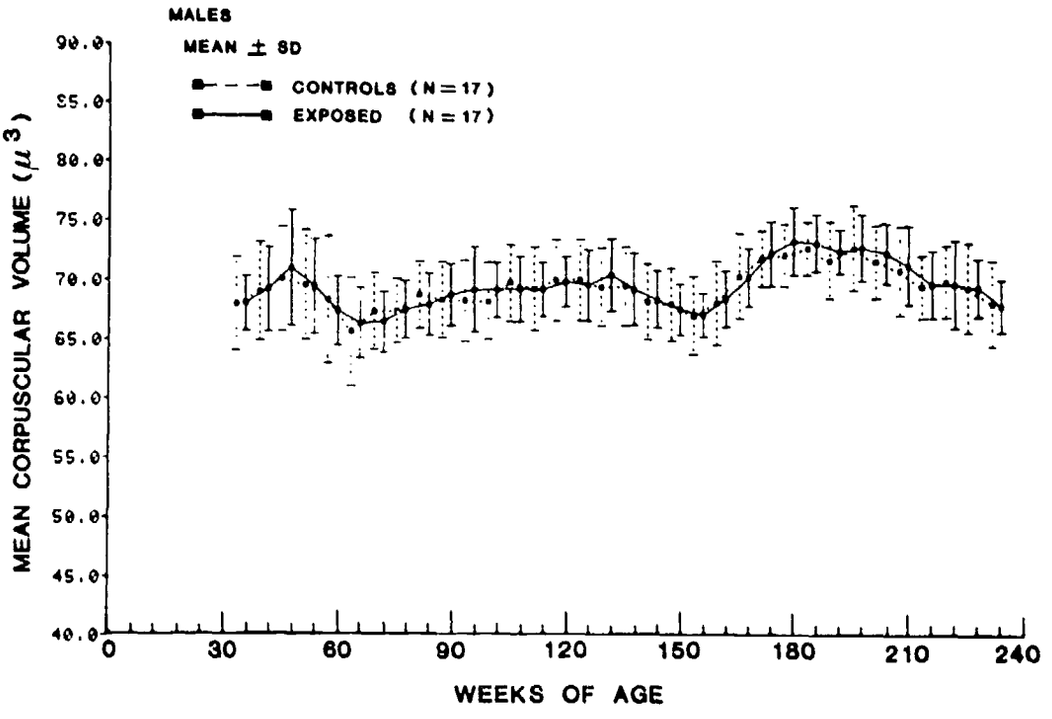


Figure 28

Hematocrit in control and ELF exposed rhesus monkeys during the first 234 weeks of life.



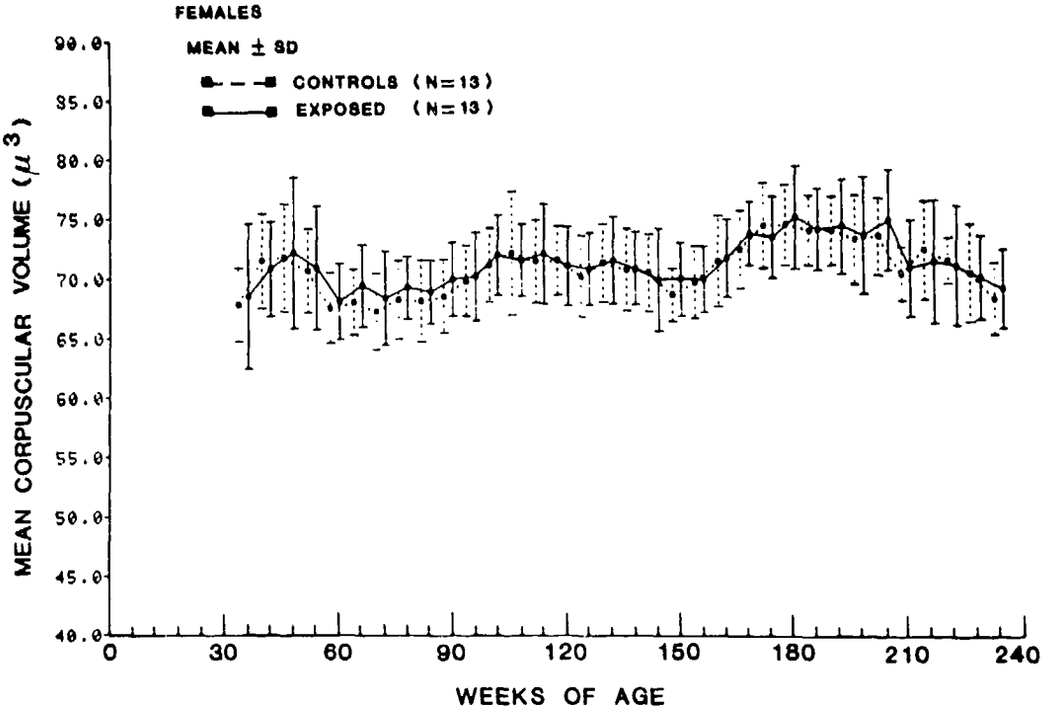


Figure 29

Mean corpuscular volume (MCV) in control and ELF exposed rhesus monkeys during the first 234 weeks of life.

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Thirty rhesus monkeys (Macaca mulatta), 17 males and 13 females, were exposed 22 hours per day, 7 days per week, from 1 to 54 months of age to extremely low frequency (ELF) electric and magnetic fields. A second group of 30 animals served as controls. The project was designed to produce fields similar to, but stronger than those associated with the Navy's ELF communications system. The field parameters (0.2 mT and 20 V/m at a frequency modulated between 72 and 80 Hz) were the same as those used in an earlier

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study in which enhanced growth rate occurred in pubescent male rhesus monkeys. The biological endpoints measured included body weight, bone growth, steroid hormones, hematology, and menstrual cycle data. No differences between exposed and control groups were observed prior to puberty. During puberty, body weight, bone growth, the age at which menarche was observed, and the progressive development of the mature endocrine rhythms of the menstrual cycle were all similar for both the exposed and control female monkeys. Body weight curves of the exposed and control males began to diverge at approximately 30 months of age, when the exposed males began to grow faster than the controls, and continued to diverge slowly through approximately 42 months of age. The growth curve separation narrowed, however, in the last 12 months of the study. The differences in growth were not statistically significant. No significant differences in mean testosterone levels of male monkeys were observed. Thus, the findings provided qualitative affirmation, but not confirmation of the prior observation of enhanced growth in pubescent male monkeys exposed to ELF fields, and failed to provide support for the hypothesis that stimulation of testosterone secretion was the cause of the growth enhancement in the initial study. Throughout the study, the ELF-exposed animals were in good health and showed no evidence of adverse physiologic effects from the exposure.